STUDIES ON IMMUNOSUPPRESSION BY SEMINAL PLASMA

Alison Jane Quayle

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Department of Surgery, University of Edinburgh

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To my parents

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Declaration

I declare that I have composed and written this thesis and that the work described in this thesis was entirely my own and performed by me, unless otherwise acknowledged.

Alison J. Quayle

December 1988

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ABSTRACT

Seminal plasma is reported to suppress a number of *in vitro* and *in vivo* parameters of immune cell function. This is believed to be fundamental to the process of reproduction, preventing sensitisation of the female to sperm antigen during coitus.

The mechanism of action of human seminal plasma (SP) mediated immunosuppression is poorly documented, and characterisation studies have been hampered by the complexity of the material. In this study *in vitro* models of T-cell, Natural killer (NK) cell and monocyte function were used to study inhibitory mechanisms of SP, and to test the contribution of major seminal plasma constituents to immunosuppression.

SP at concentrations from 0.5-4.0% final volume in culture suppressed the peripheral blood mononuclear cell (PBMC) response to phytohaemagglutinin (PHA) in a dose dependent manner. Suppression was most effective at suboptimal concentrations of mitogen and was not due to cytotoxicity or cold target inhibition. Suppression was not achieved if PBMC were preactivated for 24 hours before addition of SP, or if PBMC were preincubated in SP for 4 hours before culture with PHA. Suppression was related to decreased interleukin 2 (IL-2) receptor expression, and suppression by 1% SP was overcome by 10 U/ml recombinant IL-2.

NK cell mediated cytotoxicity against K562 target cells was also inhibited in a dose dependent manner. However, preincubation of monocytes with 1% SP before washing and culture with lipopolysaccharide(LPS)resulted in increased interleukin 1 (IL-1) production.

Seminal plasma components were characterised using a variety of techniques. A reverse phase high pressure liquid chromatography

(HPLC) separation of SP revealed 2 peaks of suppressive activity in the NK cell and proliferation assays, and these coincided with the peaks of prostaglandin E (PGE) and 19-OH-prostaglandin E (19-OH-PGE) activity. Delipidation of SP by absorption chromatography removed all NK suppressive activity, 70% of proliferation suppressive activity, and 90% of the E series prostaglandins. SP was desalted using a Sephadex G25 column, and excluded proteins separated on a DEAE HPLC column. Fractions suppressing proliferation and NK cell mediated cytotoxicity correlated with those with peak PGE activity, suggesting that some of the seminal prostaglandins were binding to seminal proteins.

Standard preparations of prostaglandins were examined at concentrations similar to those found in SP. PGE_2 (10^{-6} to 10^{-9}M) and 19-OH-PGE_1 ($10^{-6}$to 10^{-7}M) suppressed proliferation. Suppression was highest at suboptimal mitogen concentration, could not be achieved with activated cells, was reversible, was replaced with rIL-2 and correlated with decreased IL-2 receptor expression. PGE_2 (10^{-6} to 10^{-9} M) and 19-OH-PGE_1 (10^{-6} to 10^{-9} M) also inhibited NK cell mediated cytotoxicity. 19-OH-prostaglandin F_1 (19-OH-PGF_1) had little effect on proliferation or on NK cell cytotoxicity. Pure preparations of zinc and the polyamine spermidine were also examined. Zinc had little effect on proliferation when used at 10^{-5} to 10^{-8} M in culture, but spermidine (6 to 12 µg/ml) had a small but significant suppressive activity.

In conclusion, the study delineates some of the mechanisms of SP mediated immunosuppression, and suggests that the E series prostaglandins play a major role in this suppression.

CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

Semen is composed of spermatozoa, cells and a suspending fluid which is termed the seminal plasma (SP). Seminal plasma comprises 80-90% of the ejaculate and is a confluence of secretions from the prostate, seminal vesicles, epididymis, urethal and bulbourethral glands (Mann 1964). Chemical analysis of the fluid over the last century has led to the discovery and identification of a wide range of components, some of which are unique, or unique in their concentration, to semen (Table 1).

Seminal plasma is a natural diluent and transport vehicle for sperm and a number of its components have well defined roles. For example, the proteolytic enzymes play a decisive role in semen coagulation and liquefaction (Mann 1964), and high levels of fructose provide spermatozoa with the metabolic energy required for mobility and survival (Mann 1946). The physiological role of other components remains conjectural. In 1975 Stites and Erikson demonstrated that SP could suppress cellular immune responses, and proposed the *in vivo* relevance of this phenomenon which is to prevent sensitisation of females to repeated insemination. They also suggested that the presence of immunosuppressive molecules may predispose the reproductive tract to infection by organisms such as *Neisseria gonnorrheae*. In the thirteen years following this paper a number of workers have demonstrated seminal plasma mediated suppression on a wide range of immune cell functions (reviewed by James and Hargreave 1984, Alexander and Anderson 1987). Concern has also been expressed over the possible

COMPONENT	SEMINAL PLASMA CONCENTRATION			REFERENCE
IONS FREE AND BOUND	(mg/ml)			
Sodium	range	10.0 -	20.0	a
Potassium	range	5.5 -	11.0	a
Calcium	range	2.0 -	2.8	a
Magnesium	range	0.3 -	1.2	a
Chloride	range	10.0 -	20.0	a
Zinc	mean	1.4		a
Copper	mean	0.15		a
Iron	mean	0.008		a
PROSTAGLANDINS	(ug/ml)			
PGE_{1} and $_{2}$	range	2.0 -	272.0	b
19-OH-PGE_{1} and $_{2}$	range	53.0 -	1094.0	b
$PGF_{}$	range	0.1 -	7.0	b
19-OH-PGF_{1} and $_{2}$	range	3.0 -	62.0	b
POLYAMINES	(mg/ml)			
Spermine	mean	0.6		a
Spermidine	approx.	0.06		a
Putrescine	approx.	0.06		a
PROTEIN HORMONES	(mIU/ml)			
Luteinising Hormone	mean	1.34		c
Follicle Stimulating Hormone	mean	1.34		c
	(ng/ml)			
Prolactin	mean	29.5		c
Growth Hormone	mean	0.89		c
STEROID HORMONES	(ng/ml whole semen)			
Testosterone	mean	0.19		d
Dihydrotestosterone	mean	0.95		d
Androstanediol	mean	0.47		d
Dehydroepiandrosterone	mean	0.95		d
Progesterone	mean	0.26		d
Oestrodiol	mean	0.036		d
Oestrol	mean	0.067		d
OTHER	(mg/ml)			
Proteinlike Material	range	35.0 -	50.0	a
Fructose	range	2.0 -	3.0	a
Citric Acid	range	100.0 -	700.0	a
Lactic Acid	range	20.0 -	50.0	a
Lactoferrin	range	0.5 -	1.0	a
Free Amino Acid	mean	12.6		e
Phospholipid	mean	0.15		a
Pyruvic Acid	mean	30.0		a
Ascorbic Acid	mean	10.0		a

PROTEOLYTIC AND LIPOLYTIC ENZYMES
- Pepsinogen
- Chemotrypsin (like)
- Plasminogen Activator

NUCLEASES, NUCLEOTIDASES, NUCLEOSIDASES
- Prostatic Ribonuclease
- Deoxyribonuclease
- Pyridine Nucleosidase
- Nucleotide Pyrophosphatase
- 5' Nucleotidase
- Nucleosidase Phosphorylase

SEMINAL PHOSPHATASES
- Acid and Alkaline phosphatase
- Pyrophosphatase
- 3'-ATPases

GLYCOSIDASES AND GLYCOPROTEIN-GLYCOSYL TRANSFERASES
- β- Glucuronidase
- β- N-Acetylglucosomannidase
- α- Mannosidase
- α+β- Fucosidase
- β- Galactosidase

TABLE 1. HUMAN SEMINAL PLASMA COMPONENTS

References: a. Mann 1964; b. Templeton et al 1978; c. Biswas et al. 1978; d. Tea et al. 1976; e. Krampitz and Doepfmer 1962.

contribution of seminal plasma in the aetiology and pathogenesis of a wide range of genitourinary tract diseases.

The mechanism of action of seminal plasma and the primary mediators of suppression, however, still remain to be identified. The aim of this work was threefold: firstly to establish a number of *in vitro* systems to examine seminal plasma mediated immunosuppression; secondly to isolate and characterise some of the primary mediators involved in this suppression; and finally, to assess the immunological properties of these mediators.

1.2 THE MALE REPRODUCTIVE TRACT

The stepwise proliferation and transformation of the male germ cell to a mature spermatozoan is termed spermatogenesis. Many immunisation studies have demonstrated that sperm and germ cell antigens can stimulate production of sperm antibody and/or experimental autoimmune orchitis, but under normal conditions, immune responses are not detected (Brown *et al.* 1965, Alexander and Anderson 1987). In this section, the anatomy (Figure 1) and the immunological environment of the male reproductive tract and the process of spermatogenesis is reviewed.

1.2.1 The Peripheral Testis

The testis has two functional components; the seminiferous tubules and the interstitial cells. Within the testis the seminiferous tubules appear as folded loops draining at both ends into the rete testis. They are composed of a basal membrane lined by a stratified epithelium bounding the lumen. The two major cell types in the epithelium are a fixed population of Sertoli cells and a mobile population of germ cells. Various developmental stages of germ cells are present including spermatogonia (types A and B), primary spermatocytes, secondary spermatocytes, and spermatids. The three phases of spermatogenesis are: (1) mitotic division of the primary spermatogonia; (2) meiotic cell division of these sperm precursors to produce haploid spermatocytes; and (3) spermiogenesis, during which the round spermatocytes are metamorphosed into flagellated spermatozoa. The entire process takes approximately 70 days (Heller and Clermont 1964).

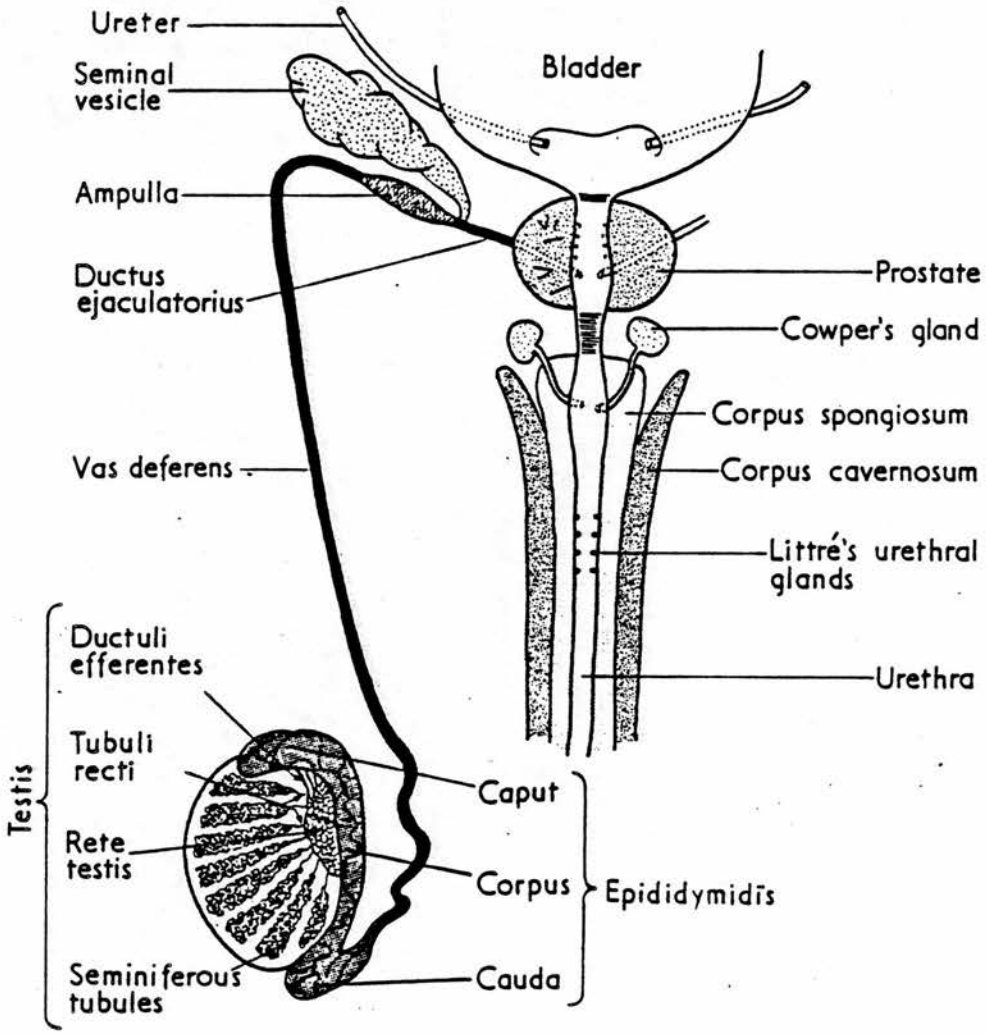

FIGURE 1. A DIAGRAMATIC OUTLINE OF THE MALE REPRODUCTIVE TRACT.

The Sertoli cells act as a supporting matrix for the germ cells, and form a continuous and complete lining within the tubular wall where they extend from the basal lamina to the lumen of the tubules. The 40 to 50 tight junctions between each pair of cells (Friend and Gilula 1972) have classically been described as the morphological site of the blood testis barrier, preventing passage of immunoglobulin, cells and complement (Gilula *et al.* 1976). HLA-DR positive macrophages are found around the seminiferous tubules and in close proximity to the outer aspect of their walls (El Demiry *et al.* 1987). Whether these macrophages are able to function as phagocytic or antigen-presenting cells remains to be established. Lymphocytes are not detected anywhere within the normal peripheral testis (El Demiry *et al.* 1987).

Interspersed between the seminiferous tubules is the interstitial tissue. This contains Leydig cells, which synthesize and secrete androgenic steroids, fibroblasts, mast cells, blood vessels, nerves, and macrophages.

1.2.2 **The Rete Testis**

Testicular spermatozoa pass through the seminal tubules into the rete testis, which is a network of canals lined by squamous epithelium. Both T-cells and HLA-DR positive macrophages are found in normal rete testis (El Demiry *et al.* 1987a). CD4 positive cells are mainly located in the interstitium, whilst CD8 positive cells which are far more numerous, are predominant in the epithelium.

The presence of CD8 positive cells may be to limit an autoimmune response to germ cells. Tight junctions in the rete are less extensive than in the seminiferous tubules, and make it more permeable to antigen and cells (Haas and Beer 1986). Further evidence for an active local immune response in the rete comes from a murine model of experimental autoimmune orchitis (Tung et al. 1988). This disease can be adoptively transferred to naive syngeneic recipients with donors enriched T-cells, and the maximum pathology is localised in the straight tubules of the rete.

The rete testis is thought to have a secretory and resorptive capacity, and the testicular plasma suspending the spermatozoa has a low dry weight and protein content. The rete also acts as a valve, permitting periodic release of sperm into the efferent ducts and movement of testicular semen towards the epididymis.

1.2.3 The Epididymis

The epididymis is a convoluted duct system of 20 metres length, and is made up of a head (caput), body (corpus) and tail (cauda). The epididymis has four functions: (a) it acts as a sperm reservoir; (b) it produces epididymal plasma; (c) it promotes maturation of sperm; (d) it disposes of ageing and superfluous sperm.

When spermatozoa enter the epididymis they have no fertilizing ability and no motility. Aquisition of these is largely due to inherent properties of the sperm, but also depends upon interactions

with the epithelium of the epididymal duct and epididymal plasma, such as deposition of epididymis-specific glycoproteins on the surface of the spermatozoa (Jones et al. 1980). The approximate transit time of epididymal sperm is twelve days. Their increased fertilising ability can be correlated to changes in morphology, chemical composition, antigenicity and metabolism (Urry 1981). The distribution of immune cells is similar to that in the rete testis (El Demiry and James 1988).

The epididymis has a high resorptive power and the dilute testicular semen is turned into more highly concentrated epididymal semen. A wide range of organic constituents are found in epididymal plasma including glycerylphosphorylcholine which is a secretory product of the epididymis itself (Dawson et al. 1957), carnitine which is probably accumulated by the epididymis from the peripheral blood, sialomucoproteins, lipoproteins, and a number of lysosomal enzymes.

1.2.4 **The Ductus Deferens**

Upon leaving the cauda epididymis the spermatozoa enter the ductus deferens, which transports sperm and its suspending fluid to the ejaculatory duct where it forms the excretory duct of the seminal vesicles. The ductus deferens consists of a vasal part which is cylindrical and has a structure similar to the cauda epididymis, and a smaller dilated area called the ampulla which is similar in morphology to the seminal vesicles. HLA-DR positive macrophages are located in the lining epithelium of both parts of the ductus deferens, and CD8 positive cells are found in the vasal area (El Demiry and James 1988).

In addition to sperm transport, the ductus deferens functions as a reservoir of sperm. Ejaculated sperm are derived from the vas and the epididymis in a 2/3 ratio.

1.2.5 **The Prostate**

The prostate is the largest male accessory gland, and is composed of alveoli lined with tall columnar secretory epithelial cells. It drains into the distal part of the prostatic urethra in close proximity to the orifices of the ejaculatory ducts allowing addition of the prostatic fluid to the other seminal components on ejaculation (McNeal 1981).

Within normal prostatic tissue, CD8 positive cells predominate and these are located in the epithelial lining of the prostate (El Demiry and James 1988). HLA-DR positive macrophages are also demonstrated in the epithelium, and CD4 positive cells and B cells in the interstitium (El Demiry and James 1988).

Prostatic secretions contribute 13 - 32% of the volume of the ejaculate (Brendler 1970). The high concentration of zinc found in semen (14mg/100 ml) is derived from prostate (MacKenzie et al, 1962), and other markers of prostate secretion include acid phosphatase and citric acid (Mann 1964).

1.2.6 **The Seminal Vesicles**

The seminal vesicles and ampulla open jointly through the ejaculatory duct into the urethral canal. The vesicles consist of a villous mucosa (epithelium and lamina propria), a layer of smooth

muscle and an external sheath of connective tissue. Two types of epithelial cells are present; secretory cells and basal cells.

Few lymphocytes are found in the seminal vesicles and these are nearly all CD8 positive (El Demiry and James 1988). No B cells and no macrophages are detected.

Secretions from the seminal vesicle form 46 - 80% of the ejaculate volume (Brendler 1970). The seminal vesicles are the major site of production of the prostaglandins (Eliasson 1959), lactoferrin, fructose and the proteins which cause coagulation in semen (Jenkins et al. 1978).

1.2.7 Antigens on Sperm

During the process of spermatogenesis, there are continual additions and deletions of cell antigens, and it is estimated that up to 50% of the antigens on mature spermatozoa were not present on spermatogonia (Romrell and O'Rand 1978). The amount of sperm specific antigens exponentially increase from approximately 4% to greater than 96% by the late spermatid stage (Romrell and O'Rand 1978). Sperm membranes not only possess sperm specific, but also HY and adsorbed antigens (Schulman 1978). Testicular germ cells (Anderson et al. 1984) and epididymal sperm do no express major histocompatibility antigens (Anderson et al. 1982).

1.2.8 **The Cellular Component of Ejaculate**

Leucocytes are found in the ejaculate of all men, but their significance remains controversial (El Demiry *et al.* 1986, Wolff and Anderson 1988a,b). A study in our laboratory found the mean leucocyte count in fertile controls (44.9 ± 25.8/high power field) significantly higher than in subfertile patients (14.6 ± 17.1/high power field), with T-cells (predominantly CD8 positive) detectable in 20% of the patients but in none of the controls. There was no correlation of white cell count with reduced semen parameters or positive microbial culture (El Demiry *et al.* 1986). In contrast, Wolff and Anderson's study (1988a) found higher numbers of leucocytes in infertile patients, with a similar distribution of immune cell types in controls and patients. A more recent report has also found that counts of more than one million cells/ml of ejaculate was significantly linked with reduced semen parameters (Wolff and Anderson 1988b).

1.2.9 **Ejaculation**

Little is known about the pre-ejaculatory phase in man. Human emission appears to be initiated by the urethral and Cowpers glands, with the prostatic secretion and spermatozoa following, and the seminal vesicle secretion delivered last. There is, however, an overlap between pre-sperm, sperm-rich and post-sperm fractions.

1.2.10 **Interactions Between Spermatozoa and Seminal Plasma**

The secretions from the male accessory glands interact with each other and with spermatozoa in a number of different ways. These include: (1) *Coagulation and liquefaction.* On ejaculation, human semen coagulates, and does not liquefy until approximately twenty minutes

later. Scanning electron microscopy reveals a dense network of fibres, separated by narrow spaces which do not allow free movement of spermatozoa. As liquefaction proceeds, amorphous material consisting of small globules appear on the fibre surface, until the fibres completely disappear (Zaneveld 1974). (2) Coating of spermatozoa. A number of seminal plasma components coat the surface of sperm. These include the ABO blood group antigens in the prostatic secretion of secreter males, a lactoferrin like substance called Weil's sperm coating antigen, and sperm agglutinating antibodies, which are discussed later. (3) Ionic and metabolic exchanges. Zinc is avidly taken up by spermatozoa (Lindholmer and Eliasson 1974), and the intracellular level of coenzymes such as ATP, NAD and cAMP is controlled by fructose, pyruvate and lactate in the seminal plasma. Carnitine from the epididymis acetylates in spermatozoa to acetylcarnitine and facilitates lipid oxidation.

1.3 THE FEMALE REPRODUCTIVE TRACT

Spermatozoa are immunologically foreign to women, but repeated insemination does not usually induce an immune response. This apparent inability to recognise sperm antigens is not explained by the priviliged immune status of the female tract. The vaginal route is adequate for immunisation against a variety of antigens (Straus 1961, Yang and Schumacher 1979) and histoincompatible grafts are uniformly rejected (Beer and Billingham 1975). In addition, antisperm antibody can be detected in a small number of women and may correlate to impairment of their fertility (Hjort 1983).

The distribution of leucocytes in the female tract has rarely been studied. An initial project undertaken in our laboratory demonstrated the presence of T-cells in the endocervical, endometrial and oviductal epithelia above the basal lamina, and between the epithelial cells, and in subepithelial connective tissue (El Demiry 1987b). The majority of these cells were CD8 positive. CD4 positive cells were few in number. Macrophages were found in the subepithelial connective tissue and these were demonstrated as HLA-DR positive. In this study B cells were rare, and only detected in the subepithelial connective tissue. In contrast, a number of studies have shown the presence of IgA and IgG containing plasma cells in the endocervix (Hulka and Omran 1969, Lippes *et al.* 1970). IgG containing plasma cells are found infrequently in uterus and vagina (Ogra *et al.* 1981).

Female reproductive tract secretions contain small but significant levels of immunoglobulin. IgA, IgM, and IgG have been detected in uterine and endometrial secretions (Moghissi 1970), cervical mucus (Moghissi 1970) and IgA and IgG in vaginal secretions (Moghissi 1970). Lactoferrin has also been located in uterine secretions (Lippes et al. 1970).

1.4 THE IMMUNOLOGICAL EFFECTS OF SEMINAL PLASMA *IN VITRO*

Literature surveys reveal that seminal plasma is reported to modulate the *in vitro* function of T and B lymphocytes, natural killer (NK) cells, polymorphonuclear neutrophils and mononuclear phagocytes (reviewed by James and Hargreave 1984, Alexander and Anderson 1987). Suppression has been demonstrated with seminal plasma from man (Stites and Erickson 1974, Marcus *et al.* 1978, Majumdar *et al.* 1982), mouse (Anderson and Tarter 1982), rabbit (Mukherjee *et al.* 1983), and bull (Prakash *et al.* 1976), and the suppression is not species specific (Prakash *et al.* 1976, Lord *et al.* 1977 and James *et al.* 1983). Effects with human seminal plasma can be demonstrated in normal and vasectomised men, and the inhibition is independent of antisperm antibodies (James and Szymaniec 1985).

1.4.1 Lymphocytes

Seminal plasma is demonstrated to suppress lymphoproliferative responses to the mitogens concanavalin A (Prakash *et al.* 1976, Lord *et al.* 1977, Anderson and Tarter 1982), phytohaemagglutinin (Stites and Erickson 1974, Anderson and Tarter 1982) and pokeweed mitogen (Lord *et al.* 1977), the antigens *Candida albicans* and tetanus toxoid (Lord *et al.* 1977), allogeneic sperm (Mukherjee *et al.* 1983) and allogeneic cells (Prakash *et al.* 1976, Lord *et al.* 1979). Inhibitory effects are not reported to be due to the loss of cell viability (Lord *et al.* 1977 Majumdar *et al.* 1982, James *et al.* 1983). Preactivated lymphocytes are less susceptible to suppression (Majumdar *et al.* 1982).

The mechanism of action of suppression of cytotoxic T-cell activity by seminal plasma is as yet unclear. Lord *et al.* (1977) report that SP inhibits the induction, but not the expression of cell mediated cytotoxicity. However, Glater and Anderson (1988) found that SP prevented destruction of cytomegalovirus (CMV) infected target cells by activated cytotoxic T-cells. This was through preventing binding of the T-cells to targets and was only seeen when the effector cells were preincubated with seminal plasma. Turner and associates (1987) also report that SP inhibits the response of cytotoxic cells to Epstein Barr Virus (EBV) infection, permitting polyclonal B cell proliferation.

Plaque forming cell assays indicate that the *in vitro* humoral response to T-cell dependent (sheep red blood cells) and independent (DNP-F, TNP-BA) antigens is reduced by seminal plasma (Lord *et al.* 1977, Thomas and Erickson 1984).

Spermatozoa themselves, when well washed of seminal plasma, are not reported to suppress lymphocyte function. Human spermatozoa show a variable, sometimes stimulatory effect in the human MLR (Stites and Erickson 1975). Autologous murine spermatozoa give a statistically insignificant slight inhibitory effect in the MLR, whilst no effect at all could be demonstrated with soluble sperm antigen (Stites and Erickson 1975). Epididymal spermatozoa did not demonstrate a suppressive effect on the *in vitro* humoral response to antigen (Thomas and Erickson 1980).

1.4.2 **Natural Killer Cells**

A number of laboratories have independently concluded that seminal plasma has a profound effect on the NK cell mediated killing of the K562 human erythroleukemic cell line (James and Szymaniec 1985, Tarter *et al* 1986, Marcus *et al.* 1987 Vallely *et al.* 1988). The inhibition of activity is recorded at all effector:target ratios used (Tarter *et al.* 1986, Marcus *et al.* 1987) and is not due to loss of cell viability (Tarter *et al.* 1986). Preactivation of the NK cells renders them far less susceptible to the seminal plasma (S. Szymaniec, personal communication). The suppressive moiety is heat stable (James and Szymaniec 1985, Marcus *et al.* 1987), and cannot be absorbed out with the K562 cells (James and Szymaniec 1985).

1.4.3 **Mononuclear Phagocyte System**

Two studies were performed on monocytes and macrophages. James and coworkers (1983) report that seminal plasma interferes with the attachment, spreading and phagocytic activity of *Corynebacterium parvum* elicited murine peritoneal macrophages and the release of reactive oxygen species from zymosan triggered human peripheral blood monocytes. Chvapil and coworkers (1977) used human prostatic fluid and extracts of dog prostatic fluid to demonstrate suppression of rat peritoneal macrophage oxygen consumption and motility.

1.4.4 Polymorphonuclear Neutrophils

Human seminal plasma inhibits release of reactive oxygen species from zymosan triggered polymorphs (James *et al.* 1983) and dog prostatic fluid inhibits oxygen consumption, hexose-monophosphate-shunt activity and phagocytosis in activated polymorphs but not resting cells (Stankova *et al.* 1976). Inhibition of antibody complement mediated killing and opsonisation of *Neisseria gonorrhoea* and other gram negative organisms by polymorphs have also been observed, but attributed to an anticomplementary activity of seminal plasma (Brooks *et al.* 1981).

1.5 THE IMMUNOLOGICAL EFFECTS OF SEMINAL PLASMA *IN VIVO*

A limited number of studies have reported the *in vivo* effects of seminal plasma in animals. Anderson and Tarter (1972) used murine seminal plasma to abrogate both primary and secondary humoral responses in mice immunised with epididymal sperm and other antigens. Preincubation of epididymal sperm with seminal plasma prior to immunisation reduced their immunogenicity, as did simultaneous injection of seminal plasma and sperm at separate sites. Hess and Bernstein (1985) have shown that in certain circumstances seminal plasma may be immunomodulatory *in vivo* and not merely suppressive: the *in vivo* responses to PHA or splenocytes from normal mice were abrogated in the presence of seminal plasma, but in mice immunised with seminal plasma, *in vitro* responses were enhanced but variable. Administration of seminal plasma rectally into rabbits is reported to depress immune responses (Richards *et al* 1984). Unpublished observations from our own laboratory suggest that seminal plasma may enhance the growth of transplanted methylcholanthrene induced tumours in syngeneic mice (K. James, personal communication).

1.6 POSSIBLE IMMUNOREGULATORY COMPONENTS IN SEMINAL PLASMA

Many of the components of seminal plasma have well-established *in vitro* and *in vivo* immunoregulatory effects, and it is highly probable that a number of them could contribute to the reported suppressive activity of seminal plasma. In Table 2, some of the molecules possible responsible for this suppression are listed, along with evidence for their involvment. The prostaglandins, ionic zinc and the polyamines are discussed in detail below as these components were examined in the course of this thesis.

1.6.1 Prostaglandins

Semen contains uniquely high concentrations of prostaglandins which derive from the seminal vesicles. Early work by von Euler (1936) had proposed the prostate as the major source, but later studies showed that prostaglandin levels correlated with fructose levels in split ejaculates (Eliasson 1959) and that seminal vesicles produce prostaglandins *in vitro* (Hamberg 1976).

Prostaglandins are 20 carbon containing fatty acids consisting of a cyclopentane ring to which is attached two chains, containing 7 and 8 carbons respectively, and are derivatives of the unsaturated fatty acid linoleic acid. Dihomo-γ-linoleic acid and arachidonic acid are incorporated in the phospholipids of cell membranes and become the precursors of the 1 and 2 series respectively. The four major prostaglandins in semen are PGE_1, PGE_2, 19-OH-PGE_1 and 19-OH-PGE_2. The 19-OH-E prostaglandins are found in concentrations up to 7.0×10^{-4} M, and the E prostaglandins up to 2×10^{-4}M (Figure 2) (Bendvold *et al.*

COMPONENT	DIRECT EVIDENCE FOR INVOLVEMENT IN SEMINAL PLASMA MEDIATED IMMUNOSUPPRESSION TECHNIQUE FOR CHARACTERISATION FROM SP OR PROSTATIC FLUID	SYSTEM SUPPRESSED	REFERENCE
PROSTAGLANDINS	Lipid extraction of SP and HPLC. Suppressive activity associated with the fractions containing 19-OH-PGE_1 and 19-OH-PGE_2.	NK cell	Tarter *et al.* (1986)
	Ion-exchange chromatography The fraction with peak suppressive activity was rich in PGE_2	NK cell	Vallely *et al.* (1988)
ZINC/ZINC PEPTIDE COMPLEXES	Prostatic fluid fractionated by Sephadex G100 chromatography. Fractions of low MW (< 5 KD) and high zinc content were suppressive, but high MW fractions with minimal zinc content were not.	Oxygen consumption and motility of peritoneal macrophages.	Chvapil *et al.* (1977)
	Affinity chromatography fractions with high affinity for zinc were strong inhibitory agents.	Oxygen consumption and motility of peritoneal macrophages.	J. Harvey (1986) (MPhil Thesis)
POLYAMINES	Unable to isolate from SP. Suppression in bovine serum supplemented cultures partially overcome by pretreatment of the serum with hydroxylamine, suggesting formation of a suppressive molecule by interaction of bovine serum polyamine oxidase with seminal polyamines.	NK cell activity suppressed in cultures supplemented with bovine serum, but not in cultures supplemented with human serum, or in serum free medium.	Vallely *et al.* (1987) (see also Allen and Roberts 1986).
PREGNANCY-ASSOCIATED PROTEIN A (PAPP-A)	Seminal plasma elution profile identical to PAPP-A. Removal of PAPP-A from seminal plasma removed suppressive activity.	Lymphocyte transformation.	Bischof *et al.* (1983)
94-KD Fc-RECEPTOR	Seminal fluid fractionated by gel exclusion chromatography. Fraction inhibitory to aggregrated IgG binding isolated by affinity chromatography on Fc coupled to CNBr-activated Sepharose 4B.	Binding of aggregated human IgG to bull spermatozoa.	Witkin *et al.* (1983)
UTEROGLOBULIN AND TRANSGLUTAMINASE	None. Treatment of prostatic fluid with antisera against uteroglubulin or transglutaminase, or an inhibitor or transglutamine (neopentyl chloroethy nitrosourea) reduced its suppressive effect.	Lymphocyte transformation (to epididymal sperm).	Mukherjee *et al.* (1983)
OPIATES	β-Endorphin immunoreactive peptides extracted with octadecysilane C8 reverse phase beads and -endorphin immunoreactivity assayed by RIA. Suppression overcome by naloxone addition.	T-Rosette formation.	Fabbri *et al.* (1985)
3KD MOLECULE	3KD molecule isolated from SP by extensive dialysis, extraction and chromatographic procedures.	Lymphocyte transformation.	Pitout and Jordaan (1976)
PROTEIN MW < 20 KD	Suppressive activity dialysed from whole seminal plasma and from the two major peaks of a Sephadex G25 separation of SP.	Inhibition of antibody complement-mediated killing and opsonisation of *N. gonorrhoeae* and other gram negative organisms.	Brooks *et al.* (1981)
PROTEIN MW > 100 KD	Sephadex G-100 chromatography of SP fraction of 1000KD contained suppressive activity.	Lymphocyte transformation.	Prakash *et al.* (1976)
	Sephadex G-25 chromatography of SP void volume contained dominant immunosuppressive activity.	Lymphocyte transformation.	Lord *et al.* (1977)

TABLE 2. SEMINAL PLASMA COMPONENTS POSSIBLY INVOLVED IN IMMUNOSUPPRESSION.

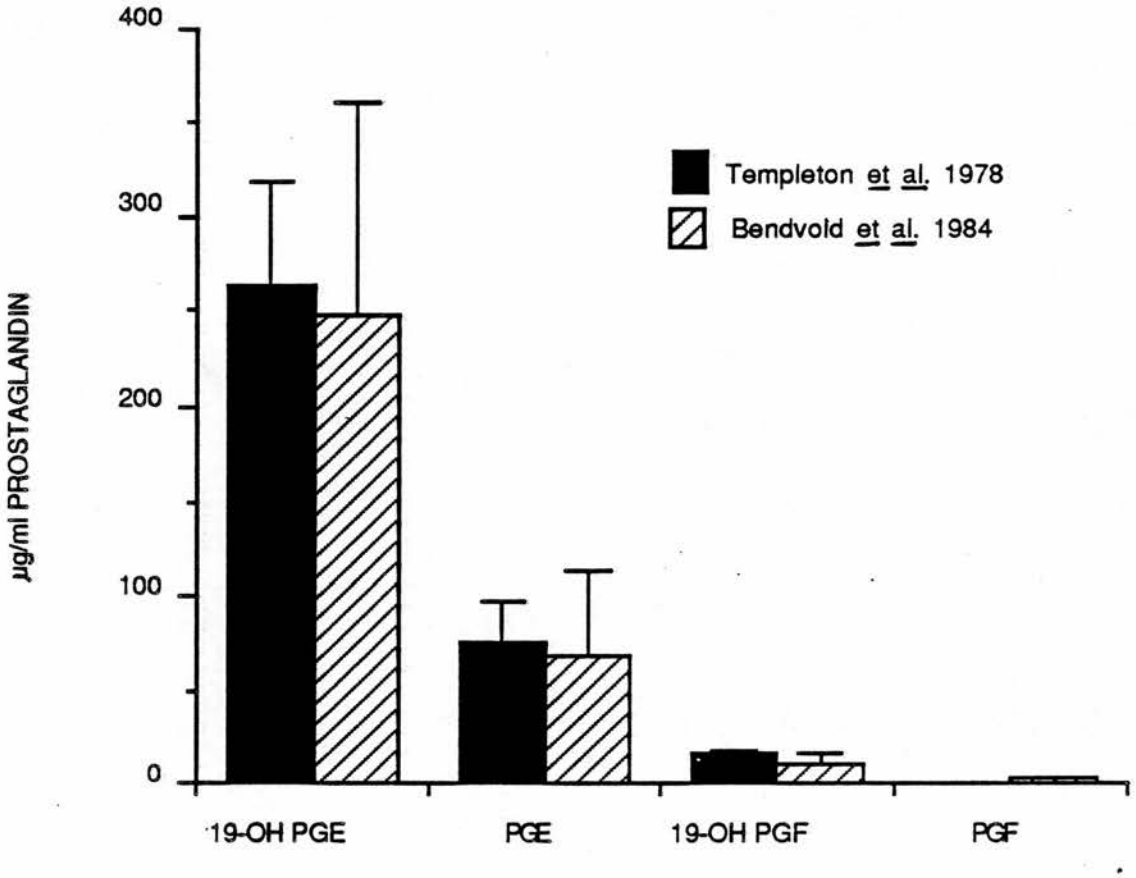

FIG 2: PROSTAGLANDIN LEVELS FOUND IN SEMEN IN TWO COMPLETE STUDIES.

1970, Templeton et al. 1978). Smaller amounts of PGF_1, PGF_2 19-OH-PGF_1 and 19-OH-PGF_2 are also present. Literature prior to 1974 cites a total of 13 prostaglandins to be found in semen, including PGA, 19-OH-PGA, PGB and 19-OH-PGB, but it is now clear that these are artifacts of storage (Middleditch 1975).

A substantial body of evidence is available recognising PGE as a major modulator of immunological and inflammatory processes. The action of PGE in controlling cell function is by a paracrine mechanism raising intracellular cAMP concentrations through receptors coupled to adenyl cyclase via G proteins (Makoul et al. 1985). Reviewing the effect of prostaglandins on lymphocytes, Goodwin and Ceuppens in 1974 concluded that PGE is a feedback inhibitor of lymphocyte proliferation, lymphokine production and T-cell cytotoxicity. More recent studies have reported that PGE exerts its antiproliferative effects through inhibition of IL-2 production (Tilden et al. 1982, Walker et al. 1983, Chouaib et al. 1985, Mary et al. 1987, Vercammen and Ceuppens 1987), IL-2 receptor induction (Vercammen and Ceuppens 1984) and transferrin receptor expression (Chouaib et al. 1985). Prostaglandin E also suppresses the NK cell mediated lysis of susceptible target cells (Droller et al. 1980, Kendall and Targan 1980).

Macrophages synthesise and release prostaglandin E in response to inflammatory and immunological stimuli (Humes et al. 1977). PGE can suppress the production of interleukin 1 and Ia expression whereas indomethacin augments these functions, suggesting that PGE acts as an autocrine regulatory molecule of macrophage immune function (Synder et al. 1982, Kunkel et al. 1986).

The immunological properties of 19-OH-PGE has so far only been implicated in one study. Tarter *et al.* (1986) report that an acid lipid fraction rich in 19-OH-PGE suppresses the cytotoxic activity of NK cells.

The role of prostaglandins in semen is still open to conjecture, but it has been postulated that they may participate in the ejaculatory process, influence sperm motility, or aid sperm transport in the female tract.

Movement of sperm in the upper reproductive tract in ejaculation would involve PG synthesis in the vas and epididymis, for which there is no evidence (Kelly 1988). Although prostaglandins affect neural transmission, the levels required are many orders of magnitude below those found in semen, and it is unlikely to be the intended purpose (Kelly 1988).

The motility of a spermatozoan is important in its fertilising capacity as certain parts of the female tract, namely the cervix and the utero-tubal junction, form a natural barrier to sperm penetration. The various prostaglandins have small but different reported effects on sperm motility. Gottlieb *et al.* (1988) found that addition of PGE_1, PGE_2 and PGF had no effect on sperm motility, 19-OH-E increased motility and PGF decreased motility. Only PGF results were significant. Conversely, Schlegel *et al.* (1981) found that PGF_2 in ejaculate negatively correlated with sperm motility and was always higher in men with disturbed fertility than in the controls.

Sperm transport in the female tract implies a passive movement of sperm due to contractile mechanisms in the female musculature. Seminal prostaglandins are active in relaxing the human uterus *in vitro* (Eliasson 1959), but in experiments where semen was deposited intravaginally, results were equivocal (Eliasson and Posse 1960). Kelly (1988) also notes that *in vivo* no vascular connection between vaginal venous drainage and uterus is known, therefore prostaglandins would have to be absorbed, and sufficient molecules escape degradation in the lungs, to affect other tissues or organs.

1.6.2 Zinc

The zinc in semen derives mainly from the prostatic secretion (Mackenzie *et al.* 1962) and there is a wide physiological range in concentration. Analysis of nearly 1500 specimens calculated a mean seminal fluid concentration of 13.3 mg/100 ml, but amounts varied from 0.1 to 62.1 mg Zn/100 ml (Eliasson and Lindholmer 1971). In human prostatic secretions and seminal plasma, zinc is loosely complexed with proteins, mostly of a glycoprotein type (Herrmann 1975). The zinc is removed by dialysis, but despite the weakness of the linkages, its binding to the cytosol is strong enough to withstand ion exchange chromatography and electrophoresis (Heathcote and Washington 1974).

A number of *in vitro* studies have been made on zinc and immune cell function. Zinc is mitogenic for human lymphocytes (Berger and Skinner 1974), and requires binding of a zinc-transferrin complex (Cunningham-Rundles and Cunningham-Rundles 1988). However, similar concentrations of zinc (0.1mM) suppress the PHA induced proliferation

of lymphocytes (Berger and Skinner 1974), the NK cell mediated lysis of K562 cells (Ferry and Donner 1984) and reduce the oxygen consumption and phagocytosis of monocytes (Chvapil et al. 1977).

In the reproductive tissues, zinc appears to be essential for normal testicular function. During spermatogenesis, zinc is first incorporated into the male gamete in the late stages of the process, with the keratanoid protein of the sperm tail binding the ion. It is suggested that zinc plays a role in sperm maturation by protecting thiol groups in this protein from excessive oxidation (Baccetti et al. 1973). Seminal plasma zinc may continue this stabilising function, as studies demonstrate that zinc concentrations in spermatozoa can be raised by incubating them in a zinc sulphate enriched medium (Eliasson et al. 1977, Lindholmer and Eliasson 1974).

1.6.3 Polyamines

Human seminal plasma is a rich source of polyamines, with a mean concentration of 60mg/100 ml of spermine detectable and around ten times less spermidine and putrescine (Mann and Lutwack-Mann 1984). If semen is left to stand, the spermine interacts with liberated phosphate from the dephosphorylation of phosphorycholine, leading to the formation of characteristic crystals. The polyamines are organic bases, and are thought to derive from the prostate (Mann and Lutwack-Mann 1984). Studies in the rat reveal the key substance in the synthesis of spermine is putrescine, a metabolite of arginine. Arginine is converted in the prostate to putrescine, via the decarboxylation of ornithine by ornithine decarboxylase. Putrescine interacts via spermidine synthase with decarboxylated S-adenosyl-methionine, yielding spermidine, and spermine is formed from spermidine

by spermidine synthetase (Williams-Ashman *et al.* 1976, Williams-Ashman and Canellakis 1979).

Information on the the immunological effects of polyamines is scarce, but there is evidence that they stimulate RNA metabolism in lymphocytes and other cells, probably at the level of RNA synthesis. Mitogenic stimulation of peripheral blood lymphocytes results in induction of ornithine decarboxylase and S-adenosyl-methionine decarboxylase (Kay and Lindsay 1973) and multi-fold increases in spermidine, spermine and putrescine in lymph node lymphocytes (Fillingame and Morris 1973). The immunological consequences of adding polyamines into lymphocyte cultures supplemented with bovine serum, which contains a polyamine oxidase, are reported in a number of studies (Tabor *et al.* 1964, Byrd *et al.* 1977, Labib and Tomasi 1981). Synthetic polyamines suppress murine lymphocyte responses to mitogen and allogenic cells in fetal calf serum supplemented cultures, but not in human serum supplemented cultures (Byrd *et al.* 1977). The suppression is not cytotoxic and is fully reversible. Conversely, bovine serum and polyamine interactions have resulted in loss of cell viability (Tabor *et al.* 1964, Labib and Tomasi 1981), and it is suggested that spermine and spermidine are converted into toxic aminoaldehydes by polyamine oxidase (Tabor *et al.* 1964), and then into acrolein (Alarcon 1970). Allen and Roberts (1986 and 1987) and Vallely *et al.* (1988) have both correlated seminal plasma mediated suppression with cytotoxicity in bovine serum supplemented cultures, and suggest much of the reported work on seminal plasma is in fact artefactual.

The exact role of polyamines in ejaculate is still debatable, although a number of suggestions have been forwarded. Spermine and spermidine are known to promote cellular growth by stimulating DNA-primed RNA polymerase. This may be important in the accessory glands themselves, but is unlikely to be operative in spermatozoa since they have weak RNA synthesizing activity. The polyamines can also covalently attach themselves to certain proteins under the influence of transglutaminases, and the post-ejaculatory clotting of rodent semen probably depends on this reaction with vesicular secretion protein (Williams-Ashman and Canellakis 1979). Spermine and spermidine have also been implicated in sperm maturation (Bamberg *et al.* 1975), protection from lipid peroxidation conferred upon cell microsomes (Kitada *et al.* 1979) and inhibition of the conversion of proacrosin to acrosin (Parrish *et al.* 1979).

1.6.4 **Others**

Many components have been implicated in the immunosuppression mediated by seminal plasma (Table 2). These include a number of other low molecular weight inhibitors besides zinc and the prostaglandins and polyamines. In the rabbit, the antigenicity of spermatozoa is reduced by the cross linking of uteroglobulin with cell surface proteins in the presence of transglutaminase (Mukherjee *et al.* 1983). Endorphin immunoreactive peptides extracted from semen (Fabbri *et al.* 1985), a small (3kD) molecule (Pitout and Jordaan 1976) and a low molecular weight protein (20kD) extracted by G-25 chromatography (Brooks *et al.* 1981) have all been reported to suppress lymphocyte responsiveness.

Prakash *et al.* (1970) and Lord *et al.* (1977) have both isolated a protein of > 100 kD from Sephadex G100 and G150 chromatography of seminal plasma which inhibited lymphocyte transformation. A large (94 kD) Fc-receptor binding protein was isolated from seminal fluid by gel exclusion chromatography, and as this inhibited binding of IgG to spermatozoa (Witkin *et al.* 1983), the authors postulate this would protect the sperm from immunological consequences of surface bound antibody *in vivo*.

1.7 SEMINAL PLASMA AND INFERTILITY

It has been estimated that up to fifteen percent of all marriages are involuntarily childless (Swerdloff et al. 1985), and that the male factor is present in or contributes to, 50% of these cases (Ross 1983). There are many causes of infertility, including physical, hormonal and immunological pathologies, but it is immune infertility which is least well understood (Prakash 1981).

Antisperm antibodies can be measured in the blood, semen and the secretions of the female tract (Rumke and Hellinger 1959, Franklin and Dukes 1964). The origin of these antibodies is unclear but much of the immunoglobulin is found in the first portion of the split ejaculate and Rumke (1976) has correlated serum and semen titres of IgG, suggesting that this class of immunoglobulin transudates from blood into the prostate. IgA concentration varies independently from serum levels, indicating a local production by plasma cells in the genital tract (Rumke 1974).

Approximately 9-10% of men in infertile marriages are reported to have significant serum levels of antisperm antibodies (Hendry et al. 1977, Hargreave 1982) and a number of studies have shown a correlation between increasing antisperm antibody titre (Rumke et al. 1974, Mathur et al. 1981, Mandelbaum et al. 1984) and antibody bound sperm (Ayvaliotis et al. 1985) with decreasing fertility. It is postulated that antisperm antibodies could interfere with fertility in a number of different ways. Antibodies may immobilise sperm in cervical mucus preventing cervical penetration (Kremer and Jager 1980);

stimulate complement mediated cell lysis (Bronson et al. 1982); enhance phagocytosis by macrophages (London et al. 1985); interfere with capacitation or acrosome reactions (Bronson et al. 1983); or induce a defective interaction with the ovum (Mandelbaum et al. 1987). Bronson et al. (1982) demonstrate that antibodies directed against sperm heads affect all of these functions, whereas those directed against tails only weakly affect mucus interactions.

The reason antisperm antibodies are produced in a small number of men and women remains unclear. In the male a breach of the blood testis barrier by infection or chemical or physical trauma may result in their production, with 40 - 60% of vasectomised men developing detectable antibody levels (Alexander and Anderson 1979). However, antibodies can also occur without histological evidence of damage (Ritchie et al. 1984). In females, it is assumed that sensitisation occurs because of an abnormal immune response by the female, or a deficiency in the suppressive activity of their partners semen. Cell mediated immune responses against spermatozoa in the male and female have not been investigated.

Seminal plasma samples have also been analysed in specific groups of patients and controls in an attempt to correlate abnormally high or low concentrations of particular components with infertility.

Several papers have implicated low levels of prostaglandins with infertility. Bygdeman and associates (1970) calculated the mean PGE concentration in normal fertile men as 34.4 ug/ml, but only 18.1 ug/ml in patients with unexplained infertility. None of the fertile group

samples had PGE concentrations of less than 11 µg/ml, but 41% of the patient samples fell below this value. Brummer and Gillespie (1974) obtained results which were not in total agreement with this study. They found no significant difference in the mean PGE levels of men awaiting vasectomy, men with unexplained infertility, and potentially infertile men with low sperm counts or motility. There was, however, a significance difference in the scatter of the first two groups. As this was due to an absence of high values in the group with unexplained infertility and a number of the fertile group had low levels of PGE, the authors single conclusion was that high levels of prostaglandin E could be associated with increased infertility.

Studies on zinc concentration and total zinc in the seminal plasma of infertile men have shown no differences in men with normal spermodiagrams, abnormal spermodiagram or those with varicocele (Sarada and Yoshida 1985). However, the percentage of men with decreased motility and normal sperm concentration was significant in the group with lower zinc concentration or decreased total amount of zinc.

A very recent study by Shohat et al. (1988) has correlated polyamine levels, antisperm antibodies and fertility. Infertile couples of at least 3 years duration and with antisperm antibodies in the sera had levels of spermine and spermidine 50% lower than controls, and a reduced ability of seminal plasma samples to induce immunosuppression.

1.8 SEMINAL PLASMA COMPONENTS AND GENITOURINARY TRACT DISEASE

Seminal plasma may play a vital physiological role in preventing sensitisation of females to spermatozoa during coitus, but a number of workers have suggested that this very role may implicate it as a factor in the aetiology or pathogenesis of a number of genitourinary tract diseases such as bacterial and viral infection and neoplastic disease (James and Hargreave 1984, Alexander and Anderson 1987). Many microorganisms are transmitted by sexual intercourse including Neisseria gonorrhoea, chlamydia, hepatitis B virus (Heathcote et al. 1974), human immunodeficiency virus (HIV) (Ho et al. 1984), herpes simplex virus (HSV) (Centifanto et al. 1972), CMV (Lang and Krummer 1972), Epstein Barr virus(EBV) (Sixbey et al. 1986) and the papilloma virus (Meisels and Fortin 1976). In vitro studies have revealed that seminal plasma inhibits the complement dependent antibody mediated lysis of Neisseria gonorrhoea and other gram negative rods (Brooks et al. 1981), the phagocytic activity of macrophages (Chvapil et al. 1977, James et al. 1983), the destruction of CMV infected target cells by cytotoxic T-cells (Glater and Anderson 1988) and the response of T-cells to EBV infection (Turner et al. 1977).

Prostatic and cervical cancer are two of the most prevalent neoplasms in men and women respectively (McGregor and Teper 1978, Bouffioux 1983). Very little work has been performed on seminal plasma and tumour growth, but data from our laboratory has suggested that seminal plasma enhanced growth of transplanted methylcholanthrene induced tumours in syngeneic mice (James and Hargreave 1984).

1.9 AIMS OF THIS STUDY

1) to establish *in vitro* models of T-cell, NK cell and macrophage function, confirm the immunosuppressive properties of whole seminal plasma and study its mechanism of action.

2) to characterise components from semen which are responsible for suppression of immune cell function.

3) to study the effect of purified components of seminal plasma on the immune response.

CHAPTER 2

MATERIALS AND METHODS

2.1 BASIC MATERIALS AND EQUIPMENT

2.1.1 Media

Unless otherwise stated, cell cultures were maintained in complete RPMI-1640 (cRPMI). RPMI-1640 medium (Gibco) was supplemented with 2mM L-glutamine (Gibco), 100 IU/ml penicillin (Gibco), 100 ug/ml streptomycin (Gibco), 5×10^{-5} M mercaptoethanol (BDH) and 10% pooled human AB positive serum (Sigma). In some experiments AB serum was replaced by 10% foetal calf serum (Flow)(cRPMI-FCS) or 10% newborn calf serum (Flow) (cRPMI-NBCS). In experiments requiring serum-free culture, HB103 serum free medium for human mononuclear cells (NEN products) was used.

2.1.2 Blood Samples

Blood samples were obtained with consent from laboratory staff or from donors to the Blood Transfusion Service, Edinburgh. Blood was collected into 10 ml sterile blood collection tubes containing 0.1 ml of a 13% solution of EDTA (Monojet). Peripheral blood mononuclear cells (PBMC) were isolated by gradient centrifugation on lymphocyte separation medium (Flow). Cells were washed x 3 with Hanks Balanced Salt Solution (HBSS) (Gibco) and resuspended to the appropriate concentration in cRPMI.

2.1.3 Seminal Plasma (SP)

Seminal plasma was obtained from routine laboratory specimens of donors attending the infertility clinic run by this department. All were negative for antibody to sperm as tested by the tray agglutination

procedure (Friberg 1974). Spermatozoa were removed by centrifugation at 1000 g for 10 minutes and pools of approximately 20 samples were made. 1 ml aliquots were stored at -20°C until used. Most batches were tested for hepatitis B surface antigen and antibodies to HIV (courtesy of the Virology Department, University of Edinburgh).

2.1.4 Mitogens and Antigens

The mitogen used throughout the study was phytohaemagglutinin (PHA) (purified grade, Sigma), dissolved in phosphate buffered saline (PBS, pH 7.2) to a stock concentration of 5 mg/ml, and stored in aliquots at -20°C until use. Lipopolysaccharide (*E. coli* serotype 055:BS, Sigma) was dissolved in phosphate buffered saline to a stock concentration of 10 mg/ml and stored in aliquots at -20°C until use. Tetanus toxoid antigen was prepared from tetanus vaccine BP (14LF/0.5ml in simple solution with 0.01% thiomersal)(Wellcome). 0.5ml of vaccine was dialysed for 48 hours against 2 litres of PBS, filter sterilised, aliquoted and stored at 4°C until use.

2.1.5 Monoclonal Antibodies (mcAbs)

Monoclonal antibodies used for studying leucocyte surface markers are given in Table 3.

Leu 2,3 and M3 were supplied as 500 µg of purified immunoglobulin in phosphate buffered saline containing 0.2% gelatin and 0.1% sodium azide. The HLA-DR monoclonal antibody was in the form of tissue culture supernatant containing 0.1% azide. Transferrin receptor and interleukin 2 receptor (IL-2R) monoclonal antibodies were supplied as tissue culture supernate with FCS and 15 uM sodium azide. All McAbs were titrated out in initial assays to determine optimum staining conditions.

McAb	CD no.	SPECIFICITY	CLONE	SUPPLIER
Leu 2a	CD8	Suppressor/cytotoxic T-cells	SK1	Becton Dickinson
Leu 3a	CD4	Helper/inducer T-cells	SK3	Becton Dickinson
Transferrin receptor	CD71	Transferrin receptor	Ber-T9	Dakopatts
IL-2R	CD25	IL-2 Receptor	ACT-1	Dakopatts
HLA-DR		Non-polymorphic class II antigens	DA6.231	Gift of K.Guy
Leu M3		Monocyte-macrophages	M0-P9	Becton Dickinson

TABLE 3. MONOCLONAL ANTIBODIES

2.1.6 **Growth Factors, Prostaglandins and Spermidine**

Human recombinant interleukin 1 (rIL-1) was obtained from Genzyme in a stock concentration of 100 Units/ml. Aliquots were stored at -20°C for a maximum of one month before use. Human recombinant IL-2 (rIL-2) was obtained from Boehringer-Mannheim at 200 U/ml and stored in aliquots at -20°C. Prostaglandin E_2 (PGE_2) (Tissue culture grade, Sigma) was dissolved in ethanol (5 mg in 1 ml), diluted with RPMI to 1×10^{-4} M, aliquoted and stored at -20°C. 19-OH-prostaglandin E_1 (19-OH-PGE_1) and 19-OH-prostaglandin F_1 (19-OH-PGF_1) (Cayman Chemicals) were made into stock solutions of 3.33×10^{-4} M and stored in aliquots at -20°C. Spermidine (free base, tissue culture grade) (Sigma) was diluted in distilled water to 10mg/ml and stored at 4°C until use.

2.1.7 **Fluorescent Microscope**

A Carl Zeiss Jena fluorescence research microscope was used.

2.1.8 **Incubator**

Unless otherwise stated all incubation procedures were carried out in a Heraeus incubator providing a constant 37°C temperature and 5% CO_2 level.

2.1.9 **Cell Harvester**

A Skatron semi-automatic cell harvester with a filterdisc transfer system was used.

2.1.10 **Counter**

β Emissions were measured using an LKB scintillometer, and γ emissions using an LKB 1260 multigamma counter.

2.1.11 **Flow Cytometer**

The flow cytometer used in these studies was the Coulter Epics C Clinical Flow Cytometer (EPICS) (Figure 3). Flow cytometry is a high precision technique which allows rapid phenotypic analysis and sorting of cells based on their physical and fluorescence associated measurements. A single cell suspension is a prerequisite of the technique. Cells are stained using a direct or indirect fluorescent technique and the preparation injected into the flow cell of the cytometer (1) where they are hydrodynamically focused (2) into single file for passage through the interrogation point. Excitation takes place either in an enclosed quartz flow cell or in air (3). Cells physically interact with the exciting beam of light, scattering it in all directions. Light scattered in the forward direction is related to size of the cell and that at 90° to the degree of refraction of its internal structure (granularity) (4). Volume and polarization

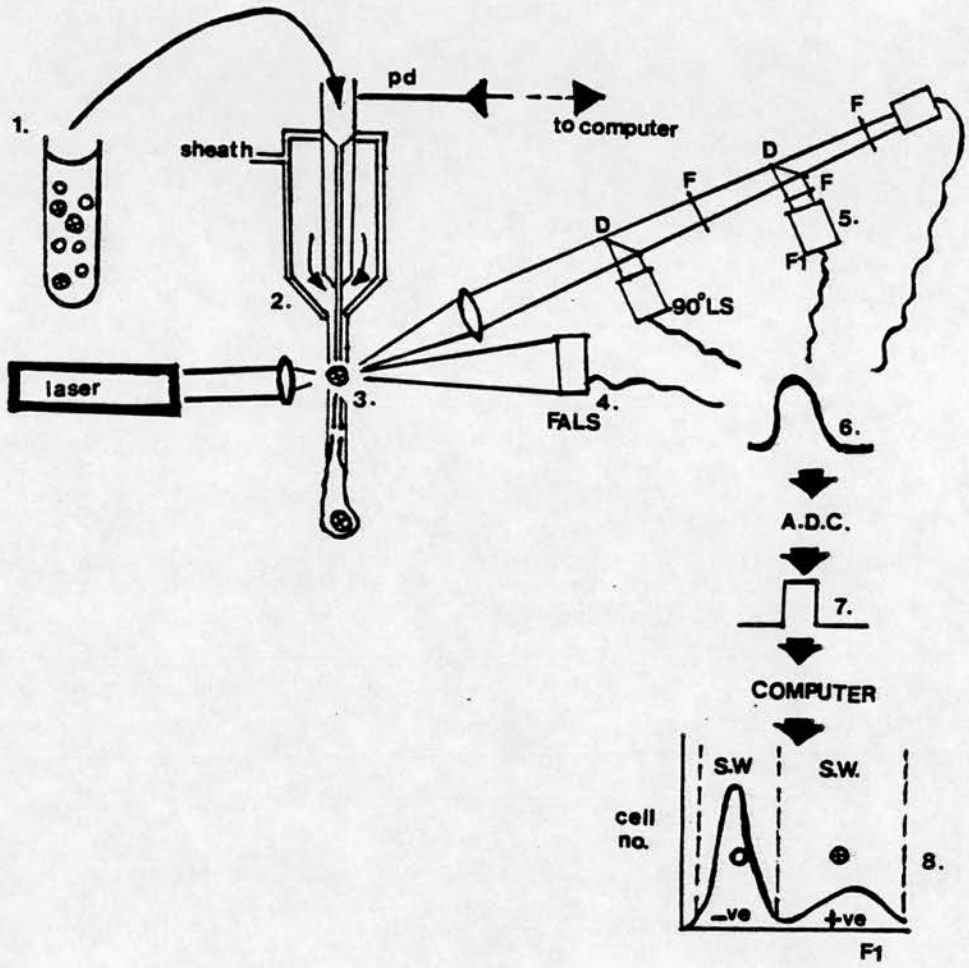

FIGURE 3. A SCHEMATIC REPRESENTATION OF FLOW CYTOMETRY.

The crossed circles represent fluorescence positive cells and the open circles fluorescent negative cells. Abbreviations used: FALS - forward angle light scatter; F1 and F2 - Fluorescence photomultiplier tube detectors; 90° LS - 90° light scatter detector; D - dichroic mirror; F - filter; pd - potential difference; ADC - analogue to digital converter.

measurements are also possible if an appropriate sizing device or polarisation filters are fitted. Excitation of the fluorochrome(s) occurs at the interrogation point (3) with the emission of light of a longer wavelength. This is also collected at 90°; selected wavelengths are directed by dichroic mirrors to photomultiplier detectors(5) and unwanted fluorescence and scattered laser light are blocked by optical filters. Analogue electric signals are generated for each particle (6) and converted to digital signals (7) for processing by computer software in order to generate one parameter or two parameter correlated histograms (8).

2.2 LYMPHOCYTE STUDIES

2.2.1 The Lymphoproliferative Response to Mitogen and Antigen

Washed PBMC were suspended in cRPMI at a concentration of 1 x 10^6/ml. 100 µl aliquots were cultured in triplicate with 20 µl mitogen and 20 µl diluted test material in 96 well round bottomed plates (Sterilin). Cultures were pulsed with tritiated thymidine (Amersham) (0.75µCi/well) after 72 hours (mitogen) or 6 days (antigen) and harvested 18 hours later with the Skatron semi-automatic cell harvester onto filter mats (Skatron). When mats were dry, each disc was placed in a closed vial with 3 ml of scintillant and counted for 60 seconds in the β-scintillometer.

Time course studies: test material was added at stated times before and after mitogen addition.

Preincubation studies: PBMC were incubated in cRPMI with test material in conical bottomed centrifuge tubes (Sterilin) for four hours. Cells were washed x 3 with HBSS before plating out with mitogen as described.

recombinant IL-2 supplemented studies: rIL-2 was added to cultures with mitogen and test material.

2.2.2 Viability Studies

These were based on a method by Parks et al. (1979) using ethidium bromide (EB) and acridine orange (AO). A 100 x stock solution was made with 50 mg of EB and 15 mg AO dissolved in 1 ml of 95% ethanol and diluted 1/100 with PBS, and frozen. 1 ml of stock was diluted 1/100 in PBS when required and stored in an amber bottle at 4°C for up to one

month. Cells were adjusted to 1 x 10^6/ml in RPMI, 20 ul mixed with 20 ul of AO/EB, and 200 cells per sample counted under the fluorescent microscope. Acridine orange enters living cells giving the nuclei a green fluorescence, and ethidium bromide is excluded by intact cell membranes, but stains the nuclei of dead cells orange fluorescent.

2.2.3 **Analysis of Cell Surface Markers and Activation Antigens**

Washed PBMC were suspended at a concentration of 1 x 10^6/ml and duplicate 1 ml aliquots were cultured with 20 ul mitogen and 20 ul test material in conical bottomed centrifuge tubes for 60 hours. Cultures were washed x 3 with HBSS and stained as follows using an indirect immunofluorescent technique. After the third wash, each pellet of cells was suspended in its residual medium and 20 ul of staining medium (RPMI + 0.1% azide + 2% bovine serum albumin). 1-10 µl of monoclonal antibody was added to one tube of each duplicate and 10 ul of PBS to the other. Tubes were incubated for 30 minutes on ice, 5 ml of HBSS added, and centrifuged at 1000 g for 7 minutes. Pellets were resuspended and a 1/20 dilution of sheep anti-mouse $(Fab)_2$-FITC antibody (Sigma) added in 15 ul staining medium. After 30 minutes cells were washed twice in HBSS and the pellets resuspended and fixed in 1 ml of 1% formaldehyde in PBS. Samples were generally stored for 24-96 hours before analysis. This does not affect any parameters being measured (Eric Miller, personal communication). Cells were analysed using the Coulter Epics C Clinical Flow Cytometer. Samples stained for second antibody only were used to define negative populations (setting an acceptable background fluorescence) and to ensure non-specific binding was not occurring in the different test situations. Samples were gated for lymphocytes using forward angle light scatter, and a minimum of 20,000 cells per sample were counted.

2.3 NATURAL KILLER CELLS

2.3.1 Natural Killer Cell Mediated Cytotoxicity Assay

PBMC were washed, resuspended to 4 x 10^6 cells/ml in cRPMI, and 5 ml aliquots were incubated in 25 cm^3 flasks (Corning) for 20 minutes at 37°C to remove adherent cells. Non adherent cells were poured off, spun at 1000 g for 5 minutes and resuspended to 2.5 x 10^6 cells/ml in cRPMI.

Target cells: The K562 human erythroleukemic line was continuously maintained in suspension culture in cRPMI + 10% FCS. Twenty four hours before an assay 5 ml of a well grown culture was added to 15 ml of fresh medium. On the day of the assay 10 mls of culture (approximately 2 x 10^6 cells) was removed to a conical bottomed centrifuge tube and washed x 3 with RPMI. The pellet of cells was resuspended in residual medium, labelled with 100 µCi ^{51}Cr (Amersham), 1 ml of RPMI added and then incubated in a 37°C water bath for 1 hour. Cells were washed and resuspended in 10 ml of RPMI for 30 minutes at room temperature to allow the release of isotope from dying cells. After a final wash, cells were suspended in cRPMI at 2 x 10^5/ml.

Chromium release assay: This was performed by adding 50 µl of target cells to 100 µl of lymphocytes and 15 µl of diluted test material. The effector to target cell ratio was 25 to 1 (unless otherwise stated) and assays were performed in triplicate. Background release was monitored using target cells and medium only, and total release was calculated from target cells lysed with 1% (v/v) Nonidet (BDH). Plates were incubated for 4 hours, centrifuged to pellet the

cells and 100 µl supernate removed for counting in the gamma scintillometer. Percentage release was calculated using the formula:

$$\% \text{ release} = \frac{\text{experiment} - \text{background}}{\text{total} - \text{background}} \times 100$$

2.4 MONOCYTE STUDIES

2.4.1 Separation of Monocytes

This was based on a method by Freundlich and Avdalovic (1983). 25 cm^3 flasks were precoated with a 2% gelatin solution and allowed to dry thoroughly. Flasks were stored in a dry incubator for up to one month before use. Donor blood was separated by the usual method, but plasma was retained and spun at 1500 g for 15 minutes. 5 ml of autologous plasma was pipetted into each gelatin coated flask, swirled around and incubated for 60 minutes. Plasma was poured off, the flasks gently rinsed, and PMBC at 3×10^6/ml in cRPMI-20% horse serum were added. Flasks were incubated for 40 minutes. NA cells were poured off and flasks rinsed with RPMI. 10 ml of a 1:1 solution of 10mM EDTA in PBS and cRPMI-20% horse serum were added to the flask, which was then tapped gently for 10 minutes. Adherent cells were poured off, spun down, and resuspended in the residual medium. The average viability of cells after this procedure was 99.0%. The average differential count from this separation procedure was 94.0% monocytes and 6.0% large lymphocytes (see below for method).

2.4.2 Testing the Purity of Monocytes

A drop of the monocyte preparation and a drop of FCS were mixed together, and cytospin preparations were made. Slides were fixed for 1 hour in acetone. Monocyte purity was examined using an indirect immunoperoxidase technique with the Leu M3 monoclonal antibody (Becton-Dickinson). Briefly, slides were rinsed in tris buffered saline (TBS) buffer (100 ml 0.2 M Tris, 165 ml 0.1M HCl, + 17 g NaCl made up to 2 litres and the pH adjusted to 7.6) for 5 minutes, followed by

an incubation in 25% normal rabbit serum (NRS) in TBS for 10 minutes. A 1/100 dilution of Leu M3 in TBS + 2% NRS was then applied for 30 minutes. Slides were rinsed with TBS, and rabbit~mouse peroxidase conjugate (1/30 dilution) in TBS+2% NRS was added for 30 minutes. Slides were washed twice with TBS. The final colour reaction was achieved by incubating preparations for 5-8 minutes in 0.01% H_2O_2 and 0.05% DAB freshly prepared in TBS. Slides were dehydrated in graded alcohol, cleared in ethylene and mounted in DPX.

2.4.3 **Production of Interleukin 1**

The method for producing and assaying IL-1 was adapted from a method by Gearing et al. (1985).

Monocytes were resuspended in HB103 serum free medium to a concentration of 2×10^5/ml. Aliquots of 1 ml were dispensed into conical bottom centrifuge tubes and 15 ul of diluted SP or medium alone were added. After one hours incubation, cells were washed x 3 with HBSS and resuspended to 2×10^5 cells/ml. Each of the treated samples was dispensed into triplicate 200 ul aliquots in 96 well round bottom plates, and 0, 1, or 10 µg of LPS / ml added. After 20 hours incubation, cells were spun down gently and supernatants pipetted off. Supernatants were passed through a 0.2 um disposable filter unit (Gelman Sciences), and stored at -70°C until use.

2.4.4 **Assay of Interleukin 1**

This was by the comitogenic mouse thymocyte assay. Endotoxin resistant C3H/He mice were used between 4-6 weeks of age. Animals were killed, the thymus removed, and a single thymocyte suspension made by

teasing the organ apart with two syringe needles. Cells were washed x 3 in HBSS and adjusted to 5 x 10^6 /ml in cRPMI-FCS (approximately 10^8 cells were obtained per animal). PHA was added at 1 µg/ml. Quadruplets of 175 µl of thymocytes were dispensed into 96 well round bottom plates. 25 µl of supernates were added in doubling dilutions from 1:2 to 1:64 (final dilutions in culture were therefore 1:16 to 1:512). A recombinant IL-1 standard curve was also prepared using doubling dilutions from 0.375-6 Units/ml. Plates were incubated for 48 hours before pulsing with tritiated thymidine (0.75 µCi/well). Cells were harvested 18 hours later onto filter discs, and counted as described earlier.

2.5 PRESENTATION OF RESULTS

Triplicate cultures were made in all lymphocyte and NK cell studies, and quadruplet cultures in the thymocyte proliferation assay. Very occasional single rogue counts were not included in the final data. All results are presented as the mean and standard deviation (standard deviations are given as horizontal bars above and below the mean in all graphs). Standard deviations in mitogen and antigen stimulated lymphocyte cultures, the thymocyte proliferation assay, and NK cell assays were less than 20%, and in baseline lymphocyte cultures, were less than 25%. Where indicated, results were statistically analysed using a paired t-test (Dr. G. Altmann, personal communication).

2.6 SEPARATION TECHNIQUES

2.6.1 Large Volume Dialysis

8/32 dialysis tubing was soaked in several changes of PBS, and 2 ml of seminal plasma was securely tied in a length of the tubing and dialysed overnight at 4°C in 4 litres of PBS. Dialysed seminal plasma (HSP_{lvd}) was stored at -20°C until use.

2.6.2 Small Volume Dialysis

8/32 dialysis tubing was soaked in several changes of PBS, and 1 ml of seminal plasmal was securely tied in a length of tubing and dialysed overnight at 4°C in 20 ml of PBS. Dialysis tubing containing buffer only was also dialysed to act as a control for culture experiments (B). Dialysed seminal plasma (HSP_{svd}) and the dialysate were stored at -20°C until use.

2.6.3 Fractionation of Seminal Plasma by Sephadex G200 Gel Filtration

Sephadex G200 (Pharmacia) was swollen by adding 10 g to 750 ml of PBS and leaving overnight. Excess liquid was removed, and the gel pipetted into a 30 x 3 cm column. Once the column was packed, PBS was pumped through at approximately 20 ml/hour using a peristaltic pump. Seminal plasma (0.5 ml centrifuged at 50 000 g for one hour) diluted in 0.5 ml PBS was applied to the column. An automated fraction collector was attached to the column and 1.5 ml fractions were collected. UV absorbing material was monitored at 280 nm, and the fractions from the three resulting peaks pooled, filtered and stored at -20°C.

2.6.4 Reverse Phase Chromatography

This procedure was carried out by Dr. R.W. Kelly of the MRC Reproductive Biology Unit, Edinburgh. A 20 ml pool of a seminal plasma was centrifuged at 50 000 g for 15 minutes, and the precipate discarded. The sample was then separated by reverse phase chromatography on a 15 x 300 mm c18 30uM silica column (Waters, Harrow) with a linear gradient of acetonitrile in water from 0 to 80%. Sixty 4 ml fractions were collected over a 4 hour period. A 0.7 ml aliquot of each fraction was removed to determine UV absorbance and 0.1 ml was diluted to 1.1 ml with a methyloximating solution and assayed by radioimmunoassay for PGE (see below). The remainder of the fractions were freeze-dried and reconstituted with RPMI-1640 (Gibco). Aliquots were frozen at -20°C until use.

2.6.5 Delipidation of Seminal Plasma by Absorption Chromatography

This procedure was carried out by Dr. R.W. Kelly. A 20 ml pool of seminal plasma was centrifuged at 50 000 g for 15 minutes, and the precipitate discarded. 2 ml of the sample was delipidated by passage through a C18 reverse phase cartridge (Seppack-Waters) which had been primed with methanol. A small sample of the eluate was measured for PGE and 19-OH-PGE (see below), and the remainder of the sample frozen in aliquots at -20°C until use.

2.6.6 Anion Exchange Chromatography

The procedure was carried out by Dr. R.W. Kelly. A 20 ml pool of seminal plasma was centrifuged at 50 000 g for 15 minutes, and the precipate discarded. 2.5 ml of the sample was loaded onto a PD10

Sephadex G25 column (Pharmacia) which had previously been washed with 20 ml water. The excluded protein was collected in 3.5 ml water and 2.0 ml of this eluate together with 2.5 µCi of a PGE_2 tracer (Amersham) was injected into a 7.5 x 75 mm DEAE 5PW HPLC column (Waters). The column was equilibrated with 20 mM ammonium formate (pH 7.6) (buffer A) and eluted at 3 ml/min with a gradient to 100% of buffer B (1.0 M ammonium formate). Protein distribution was determined by UV absorption at 280 nM and 2 min fractions were collected. Distribution of labelled PGE was assessed by scintillation counting. The PGE content of each fraction was determined by mixing 200 ul aliquots of each fraction with methyloximating solution and measuring after suitable dilution in the PGE_2 radioimmunoassay (see below). The remainder of each fraction was freeze-dried and reconstituted with RPMI-1640 (Gibco). Aliquots were frozen at -20°C until use.

2.6.7 **Radioimmunoassay of PGE and 19-OH-PGE**

This was carried out by Dr. R.W. Kelly and Ms. P. Holland. Radioimmunoassays were carried out on prostaglandins protected as methyl oximes, and has been described in detail elsewhere (Kelly et al. 1984). Antisera was raised against the methyloximated prostaglandin, and methyloximated standards were used to create the standard curves. For the 19-OH-PGE assay the prostaglandin used to raise the antiserum was extracted from human semen and the antiserum cross reacted equally with 19-OH-PGE_1 and 19-OH-PGE_2. The cross reactivity against PGE_2 and PGE_1 was less than 1% although there was appreciable (5%) cross reactivity with 19-OH-PGF. The latter was not considered a problem given the lower amounts of 19-OH-PGF found in semen (Templeton et al.

1978). In the PGE_2 assay the cross reactivity against PGE_1 was 26% and against 19-OH-PGE_1 1%. The tracers used were a ^{125}I labelled peptide coupled through proline to PGE 2 methyloxine for the assay and a similar conjugate with tyrosine methyl ester for the 19-OH-PGE methyl oxine assay. Separation of bound and free tracer was achieved by magnetic separation of second antibody coupled to magnet latex particles (Amerlex, Amersham).

CHAPTER 3

RESULTS

3.1 MECHANISMS OF ACTION OF WHOLE SEMINAL PLASMA ON IMMUNE CELL FUNCTION

3.1.1 The Lymphoproliferative Response to Mitogen

Lymphocyte proliferation may be induced by plant lectins such as phytohaemagglutinin (PHA), and can be measured by assessing incorporation of a radiolabelled precursor of DNA (usually tritiated thymidine) into dividing cells. Unless stated otherwise, DNA synthesis was assessed in PBMC cultures 90 hours after initiation of culture, with addition of tritiated thymidine 18 to 20 hours before harvesting.

A comparison of fresh and frozen seminal plasma pools, and of different frozen seminal plasma pools.

Practicality dictated that seminal plasma samples routinely tested were stored at -20°C for a number of months during use. It was therefore necessary to establish that these frozen samples behaved in a similar way to fresh semen samples. A 90 hour lymphocyte proliferation to PHA was performed with the addition of either a frozen SP pool (n = 30 donors) which had been stored for approximately 2 months at -20°C and frozen-thawed twice, or a fresh SP pool (n = 20 donors) which was used within 6 hours of collection. Cultures were stimulated with 0, 1.25, 5 or 10 µg/ml PHA in the presence of 1, 1 or 2% SP (v/v).

The results demonstrate that SP has little effect on basal proliferation (± 15%) and the suppressive effect decreases with higher concentrations of PHA. This is discussed in detail from page 55. Statistical analysis of the results with fresh and with frozen SP was made using a paired t-test. Eight pairs were constructed using the proliferation results obtained with the four mitogen concentrations and 1 and 2% SP. No significant difference was found between the pools ($p > 0.5$).

It was also necessary to demonstrate that the four frozen SP pools used in this study all had a similar pattern of activity. The pools could not be tested in a single assay as they were used sequentially over 18 months, but Figure 4 illustrates the results for four proliferation assays to 5 and 10 µg/ml PHA using the same PBMC donor and 2% SP (v/v). As the standard deviation bars overlap, we can conclude there is no difference in the activity of the pools except that due to sampling error.

(%SP (v/v) IN CULTURE)	CPM + SD (% SUPPRESSION OF CONTROL RESPONSE GIVEN IN BRACKETS)			
	0 µg/ml PHA	1.25 µg/ml PHA	5.0 µg/ml PHA	15.0 µg/ml PHA
0 (control)	486 ± 56	6019 ± 910	36017 ± 910	50241 ± 4488
1.0% Fresh SP	432 ± 112	1297 ± 96 (78.5)	4468 ± 584 (57.6)	47733 ± 784 (5.0)
1.0% Frozen SP	405 ± 11	1597 ± 184 (78.5)	5534 ± 220 (54.6)	40110 ± 3497 (20.2)
2.0% Fresh SP	555 ± 37	712 ± 152 (88.2)	2241 ± 281 (93.8)	22024 ± 2001 (56.2)
2.0% Frozen SP	532 ± 119	847 ± 68 (86.0)	4605 ± 449 (87.3)	29779 ± 322 (40.9)

Table 4. A COMPARISON OF THE EFFECTS OF FRESH AND FROZEN SEMINAL PLASMA ON THE LYMPHOPROLIFERATIVE RESPONSE TO PHA.

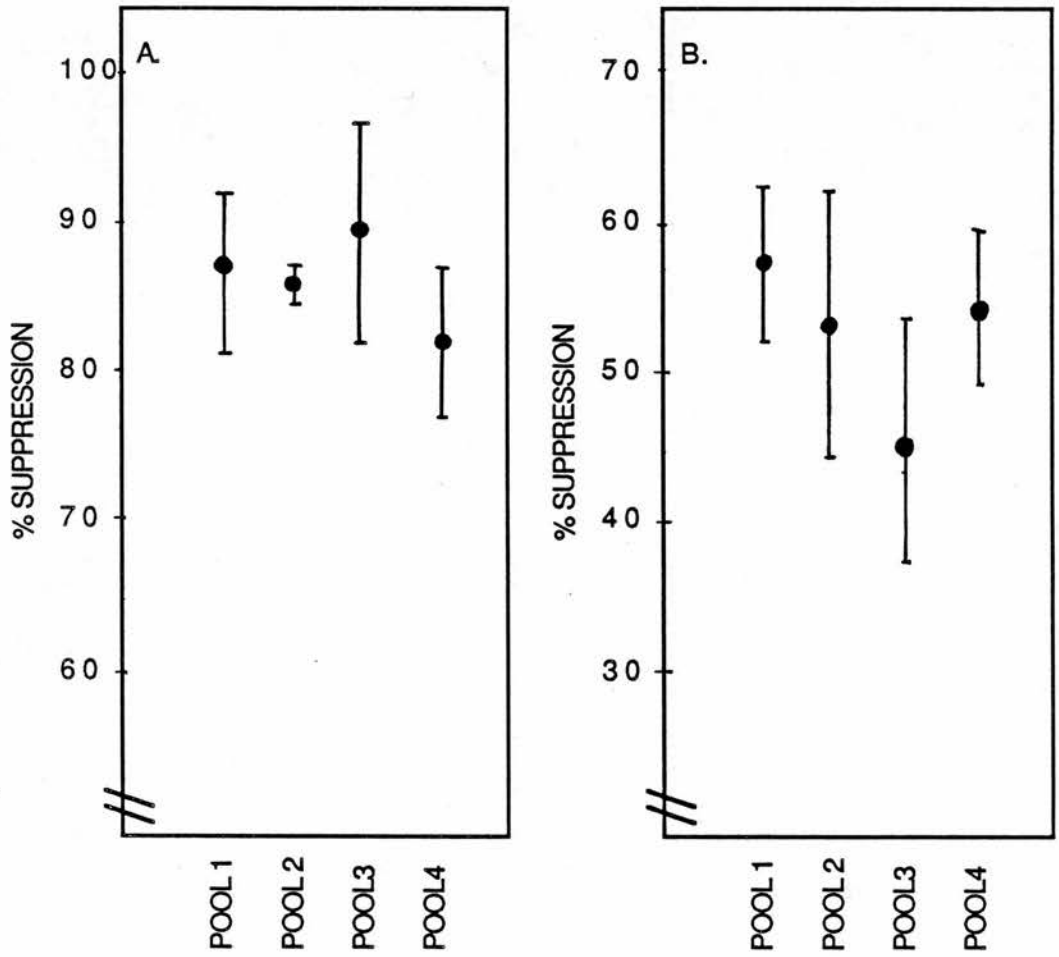

FIG 4 : A COMPARISON OF THE EFFECT OF FOUR DIFFERENT FROZEN SEMINAL PLASMA POOLS ON THE LYMPHOPROLIFERATIVE RESPONSE TO PHA.

In four separate experiments,PBMC from the same donor were cultured for 90 h ours with 5µg/ml PHA (4A.) or 10µg/ml PHA (4B.) and medium alone or 2% SP (v/v) from one of the four pools. Results are expressed as the % suppression of the control culture in the absence of SP. Control responses in cpm ± sd were: 42415 ± 6255; 23311 ± 491; 23158 ± 3275; & 30936 ± 5537 at 5µg/ml and 44680 ± 558; 66691 ± 5987; 38597 ± 5220 &38407 ± 2373 at 10µg/ml.

A comparison of PBMC donors.

In addition to ensuring that different SP pools behaved similarily, it was important to establish that the proliferative responses of different PBMC donors were suppressed in a similar way. Figure 5 illustrates the results obtained in four 90 hour proliferation assays with four different donors to 1.25, 5 and 15 µg/ml PHA in the presence of 2% SP (v/v). Although control donor responses to PHA varied considerably (data not given here, but see throughout results section), SP suppressed proliferation in each donor by a similar value. Standard deviation bars overlap at each mitogen concentration, and we can conclude there is no difference in the effect of SP on different PBMC donors, except that due to sampling error.

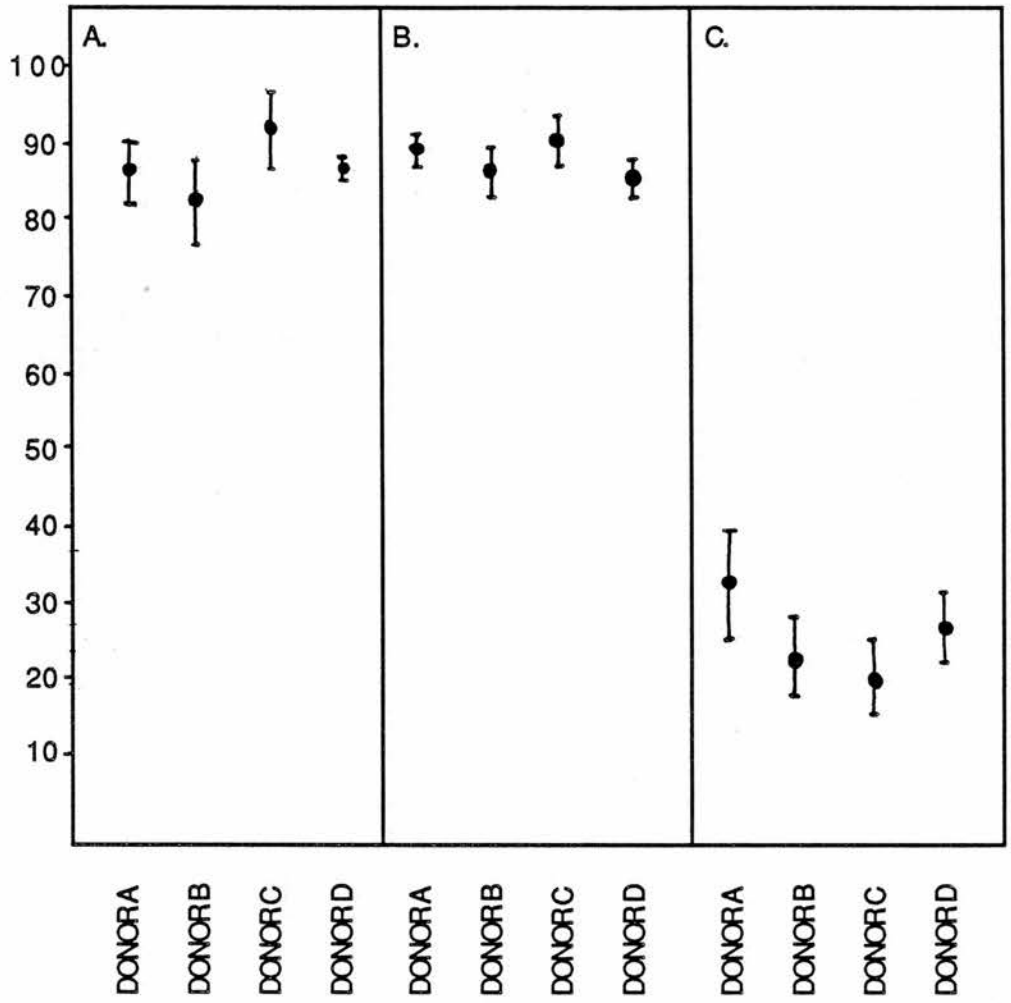

FIG 5 : A COMPARISON OF THE EFFECT OF SEMINAL PLASMA ON FOUR PBMC DONORS.

PBMC from four donors were cultured for 90 hours with 1.25 (5A.), 5.0 (5B.) or 15.0 (5C.) µg/ml PHA and 2% SP (v/v). Results are expressed as the % suppression of the control culture in the absence of SP. The controlvalues for donors A, B, C and D respectively were (cpm ± s.d.): 6019 ± 910, 7893 ± 844, 9854 ± 624 & 16538 ± 1423 at 1.25 µg/ml PHA; 36017 ± 920, 19833 ± 1264, 49712 ± 1793 & 51194 ± 1035 at 5.0 µg/ml and 5024 ± 488, 34724 ± 3873, 70379 ± 249 & 98724 ± 9944 at 15.0 µg/ml.

Dose response curves and viability of SP treated cells.

The 90 hour proliferative response to a range of PHA concentrations (1.25 - 40 ug/ml) in the presence of 1 or 2% (v/v) seminal plasma in culture is illustrated in Figure 6. Suppression mediated by 1 and 2% SP is maximal at the lowest PHA concentration used in this experiment, and decreases with increasing mitogen dose. At a post optimal PHA concentration, suppression by 1% SP is completely abrogated.

In Figure 7 a seminal plasma dose response curve to three suboptimal concentrations of PHA is shown. Suppression is again maximal at the lowest mitogen dose, and SP is effective from 0.25-4% (v/v) in culture. Viability studies were made on PBMC exposed to These concentrations of SP for 90 hours with no mitogen present (Table 5). Using ethidium bromide which stains non-viable cells orange, and acridine orange which is a vital stain, only a very small loss of viability was demonstrated. This was < 4% with SP used between 0.5 - 2%, and 9% when SP was used at a final concentration of 4% in culture.

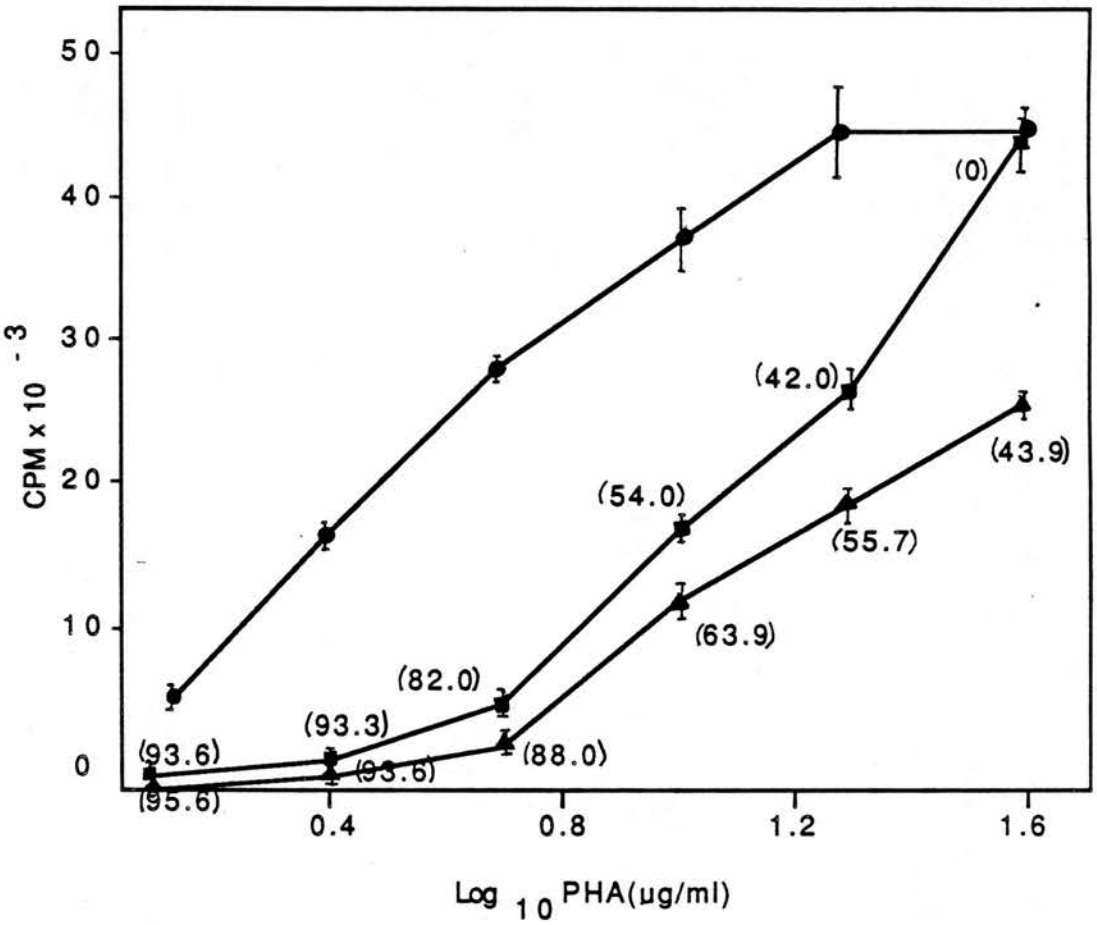

FIG 6 : THE EFFECT OF SEMINAL PLASMA ON THE LYMPHOPROLIFERATIVE RESPONSE TO INCREASING CONCENTRATIONS OF PHA.

PBMC were cultured for 90 hours with 1.25 - 40.0µg/ml PHA and medium alone(●),1% SP(v/v)(■) or 2% SP(v/v)(▲). The figures in brackets are the % suppression of the control response without SP.

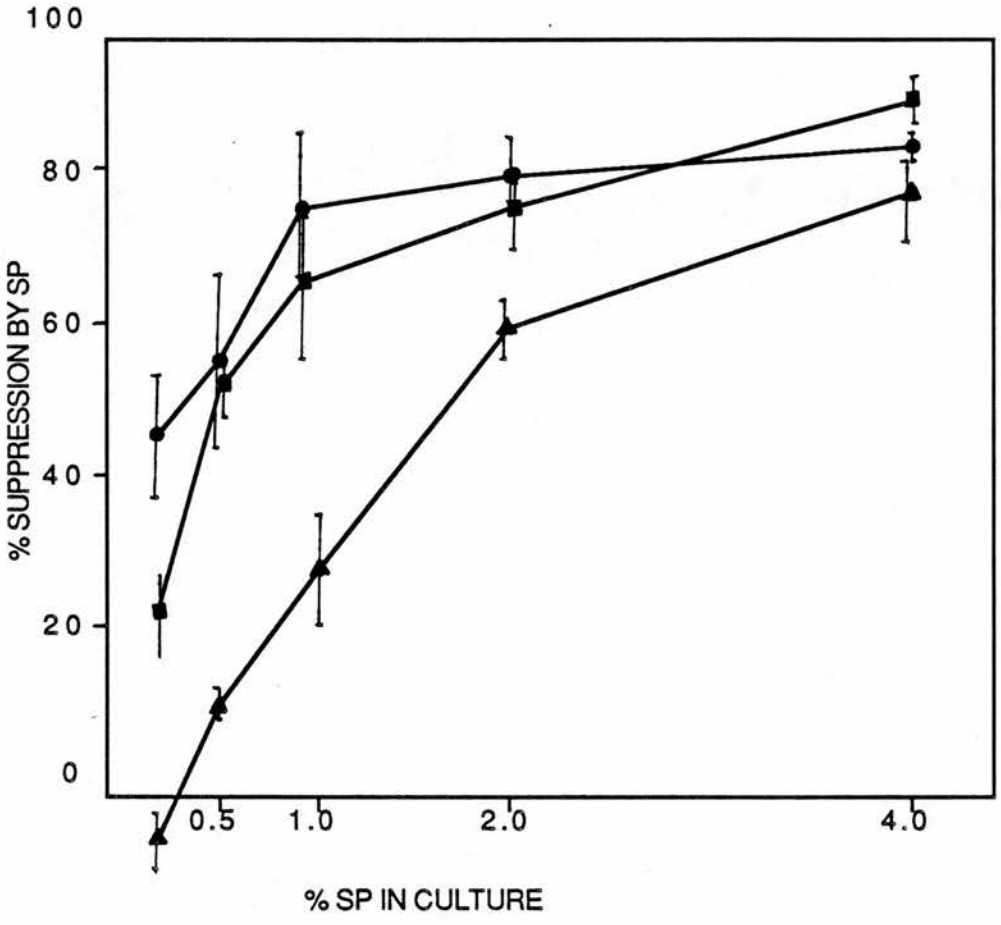

FIG 7 :THE DOSE-RESPONSE EFFECT OF SEMINAL PLASMA ON PHA INDUCED LYMPHOPROLIFERATION.

PBMC were cultured for 90 hours with 1.25 (●), 5.0 (■) or 10.0 (▲) µg/ml PHA and medium alone or 0.25-4% SP(v/v). Results are expressed as the % suppression of the control response without SP. (Control responses in cpm ± sd were: 4787 ± 251 at 1.25 µg/ml PHA; 37951 ± 2552 at 5µg/ml PHA; and 41924 ± 184 at 10 µg/ml PHA.

%SP (v/v) IN CULTURE	% VIABLE CELLS
0	98.4
0.5	94.5
1.0	95.7
2.0	96.0
4.0	89.3

TABLE 5. THE VIABILITY OF CELLS AFTER 90 HOURS IN CULTURE WITH SEMINAL PLASMA.

Viability was assessed using ethidium bromide and acridine orange and 200 cells were counted per sample.

Reversibility of suppression.

A preincubation study was next initiated to discover if pretreatment with seminal plasma could suppress subsequent responses to mitogen. PBMC were incubated with or without 1% SP (v/v) for four hours at 37°C, washed three times and then cultured with PHA for 90 hours (Table 6). At all concentrations of PHA, proliferative values of SP pretreated cells were equal to those of the SP untreated cells, suggesting complete reversibility of suppression.

PHA µg/ml	CPM + SD	
	CONTROL CELLS	1% SP PRETREATED CELLS
0	437 ± 57	487 ± 44
1.25	6088 ± 871	6888 ± 2090
5.0	28023 ± 2158	29867 ± 233
10.0	24772 ± 1206	24308 ± 1520

TABLE 6. THE EFFECT OF PRETREATING LEUCOCYTES WITH SEMINAL PLASMA.

PBMC were pretreated with medium alone or 1% SP (v/v) at 37°C for 4 hours before culture with PHA for 90 hours.

Time course studies.

A time course assay was established to examine at which time points in the process of activation and differentiation SP could mediate its effects. SP 1% (v/v) was added to cultures at 4 and 24 hours prior to, at the same time, or 4, 24 or 72 hours after PHA. Two suboptimal concentrations of PHA were used, and cultures harvested after 90 hours (Figure 8). Suppression of proliferation was maximal when SP and mitogen were added together or when SP was added 4 hours before mitogen. Addition of SP 24 hours before or 4 hours after PHA resulted in a small(<20%) decrease in suppression. If SP addition was delayed until 24 or 72 hours after initiation of cultures with PHA, virtually no suppression of proliferation was demonstrated (< 7.0%).

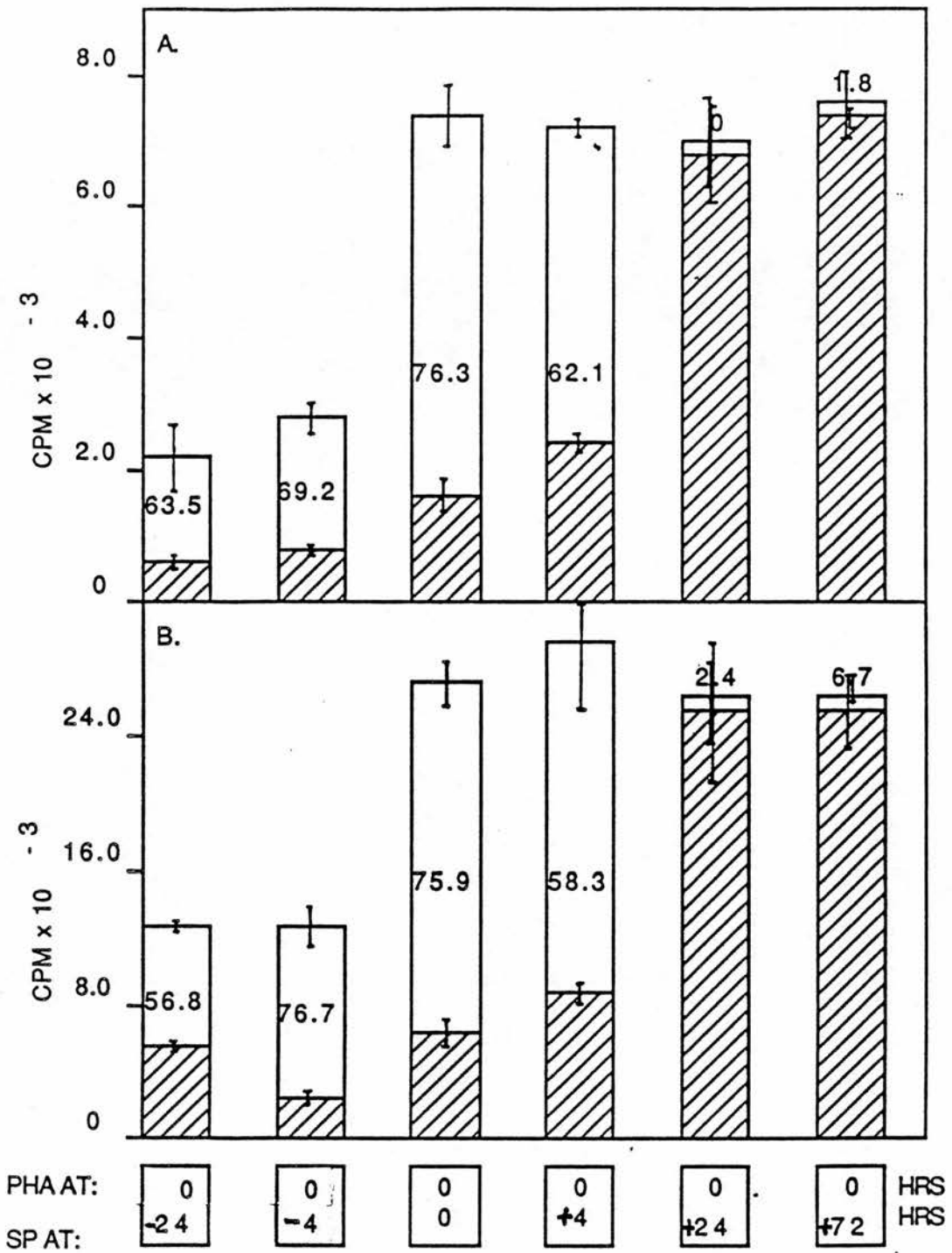

FIG 8: THE EFFECT OF TIME OF ADDITION OF SEMINAL PLASMA ON THE LYMPHOPROLIFERATIVE RESPONSE TO PHA.

PBMC were cultured for 90 hours with 0.6(8A.) or 5.0 (8B.) ug/ml PHA. 1% SP(v/v) ▨ , or RPMI □ were added at the same time, 24 and 4 hours before, or 4,24 or 72 hours after mitogen. Figures given are the % suppression of the control response without SP.

Expression of the IL-2 receptor.

Induction of T-cell proliferation first requires that the T-cells are activated to express receptors for interleukin 2 and subsequently that they respond to IL-2 by proliferation and that they produce this growth factor (Cantrell and Smith 1984). A study was therefore initiated to look at the correlation between receptor induction and proliferation in PHA stimulated cultures under the influence of SP. IL-2 receptor expression was measured using a monoclonal antibody to the receptor in an indirect immunofluorescence staining technique, with subsequent analysis of cells using a flow cytometer. Initial experiments with control cultures showed that when PHA induced IL-2 receptor expression was measured over a few day period, maximum expression occurred between 48 and 72 hours (data not given), therefore, all subsequent experiments measured receptor expression at 60 hours.

Table 7A illustrates the result of culturing PBMC from 2 donors with 0.25 or 10 µg/ml PHA and 2% seminal plasma (v/v). Parallel cultures were set up to measure receptor expression and proliferation (these cultures were pulsed at 48 hours and harvested at 60 hours). The number of freshly prepared PBMC expressing receptors for IL-2 and the CD4 and CD8 antigens was analysed to verify that donors values fell within the normal range. In 60 hour cultures with no added mitogen, the number of cells expressing IL-2 receptors in both donors was similar to the number of freshly separated PBMC, and SP had no effect on expression. In donor A, SP

DONOR	PHA ug/ml	% CELLS POSITIVE FOR IL-2R: CONTROL	% CELLS POSITIVE FOR IL-2R: 2% SP (v/v)	% SUPPRESSION OF CONTROL	CPM + SD CONTROL	CPM + SD % SP (v/v)	% SUPPRESSION OF CONTROL
A	0	2.8	2.6	7.2	132 ± 27	187 ± 3	0
A	2.5	25.5	9.2	64.1	19471 ± 753	5184 ± 116	73.3
A	10.0	61.3	38.1	37.9	30722 ± 831	20463 ± 104	33.3
B	0	3.8	3.9	0	173 ± 21	155 ± 18	10.5
B	2.5	46.3	14.6	68.5	14154 ± 908	1281 ± 218	91.9
B	10.0	52.6	32.7	37.8	17466 ± 401	12500 ± 2591	28.5

TABLE 7A. THE EFFECT OF SEMINAL PLASMA ON IL-2 RECEPTOR EXPRESSION AND LYMPHOPROLIFERATION.

PBMC from 2 donors were cultured for 60 hours with 0, 2.5 or 10 ug.ml PHA and medium alone or 2% SP (v/v). Donor A fresh PBMC values were : IL-2R = 1.7%, CD4 = 30.1%, CD8 = 11.4%. Donor B: IL-2R = 1.8%, CD4 = 17.1%, CD8 = 11.5%.

decreased the number of cells expressing the IL-2 receptor in stimulated cultures, and suppression was more effective at the lower PHA concentration. In all the experimental conditions, receptor expression paralleled the values for proliferation. Similar results were achieved with donor B, except for the disproportionately large decrease in proliferation at the lower PHA concentration used. The proliferation results obtained in these studies also confirm that the pattern of suppression by seminal plasma is the same after 60 hours of culture as 90 hours of culture.

It was not possible to present any statistical analysis of the receptor studies. Duplicate cultures were always made, but one was stained for second antibody only to provide a control for each treatment. Table 7B, however, illustrates the effect of 2% SP (v/v) on IL-2 receptor expression in five separate experiments using six PBMC donors stimulated with 2.5 and 10 µg/ml PHA. Suppressive effects were remarkably consistent with standard deviations of less than 5%, and indicate that the single results obtained in these studies are reliable, and that suppressive effects of seminal plasma are similar in all donors.

		% SUPPRESSION OF CONTROL IL-2 RECEPTOR EXPRESSION BY 2% SP (v/v)	
EXPERIMENT	DONOR	2.5 µg/ml PHA	10 µg/ml PHA
1	A	64.1	37.9
	B	68.5	37.8
2	C	74.3	ND
3	D	67.4	37.3
4	E	ND	36.0
5	F	77.7	47.3
		$\bar{x}$ = 70.4	$\bar{x}$ = 39.3
		SD = 4.9	SD = 4.0

Table 7B THE EFFECT OF SEMINAL PLASMA ON IL-2 RECEPTOR EXPRESSION IN SIX PBMC DONORS.

In five experiments, PBMC from six donors were cultured for 60 hours with 2.5 or 10 µg/ml PHA and medium alone or 2% SP (v/v). Results are expressed as the percentage suppression of IL-2 receptor expression of the control values (at 2.5 µg/ml PHA these values were 25.5, 46.3, 25.3, 44.4 and 37.5 % cells positive for IL-2R in donors A, B, C, D, F, respectively, and at 10 ug/ml PHA 61.3, 52.6, 59.1, 21.5 and 49.9 % cells positive for IL-2R in donors A, B, D, E, and F, respectively).

Addition of recombinant IL-2

In lymphocyte cultures where endogenous IL-2 production is deficient, addition of an exogenous source has been shown to overcome this, resulting in an increased proliferation similar to controls (Palladino and Welte 1984, Farmer et al. 1986)

In this assay, a recombinant IL-2 preparation (2 or 5 U/ml) was added at the initiation of culture with 0, 1 or 2% SP (v/v) and 0, 0.6, 5.0 or 10.0 µg/ml PHA (Table 8). Results are expressed as a ratio of the control counts divided by the counts observed in the presence of SP, hence a value of 1.0 would indicate that the suppressive effects of SP had been overcome.

In control cultures, 2 U/ml rIL-2 increased the basal proliferative response and the response to 0.6 µg/ml PHA over twofold. Slightly greater values were obtained with 10 U/ml rIL-2. At the two higher concentrations of mitogen, very small effects of rIL-2 were observed (< 10% stimulation). In the presence of 1% SP a proportionately greater stimulation of proliferation was observed at all mitogen concentrations with rIL-2 addition, and with 10 U/ml virtually no suppression occurred. Similar but smaller proportionate increases were observed with 2% SP and rIL-2, and suppressive effects were not totally overcome. Greater concentrations of IL-2 were not tested.

%SP (v/v) IN CULTURE	CPM 0 PHA	0.6 ug/ml PHA	CONTROL CPM / TEST CPM	5.0 ug/ml PHA	CONTROL CPM / TEST CPM	10 ug/ml PHA	CONTROL CPM / TEST CPM
				+ 0U IL-2 /ml			
0	213	2421		15047		21175	
1	247	447	0.18	2577	0.17	9716	0.46
2	196	385	0.16	2308	0.15	8704	0.41
				+ 2U IL-2/ml			
0	443	6187		16067		22491	
1	484	4824	0.54	11625	0.66	21382	0.88
2	355	3627	0.40	9190	0.52	16623	0.68
				+ 5U IL-2/ml			
0	1275	7355		16193		26262	
1	1323	7283	1.0	15303	0.95	ND	ND
2	756	4394	0.60	11820	0.73	19030	0.73

TABLE 8. THE EFFECT OF RECOMBINANT IL-2 ON SEMINAL PLASMA INDUCED SUPPRESSION OF LYMPHOPROLIFERATION.

PBMC were cultured for 90 hours with PHA (0, 0.6, 5.0 or 10.0 ug/ml), 0, 1 or 2% SP and 0, 2 or 5 U/ml recombinant IL-2. Standard deviations are not given, but were all less than 15% (mitogen stimulations) or 20% (baseline values).

3.1.2 **The Lymphoproliferative Response to Antigen**

PBMC from a known responder were cultured for seven days with tetanus toxoid, with or without 1% SP (Table 9). SP suppressed the proliferative response to tetanus toxoid at all concentrations of antigen tested, and a small decrease in suppression was noted with increasing antigen dose. Similar results were obtained from one other known responder.

FINAL CONCENTRATION OF TT IN CULTURE Lf/ml	CPM + SD		% SUPPRESSION OF CONTROL RESPONSE
	CONTROL	1% SP (v/v) IN CULTURE	
0	592 ± 62	660 ± 91	0
0.08	2473 ± 255	800 ± 150	67.7
1.3	4942 ± 919	2154 ± 302	56.5
4.0	4032 ± 677	2286 ± 565	43.3

TABLE 9. THE SEVEN DAY LYMPHOPROLIFERATIVE RESPONSE TO TETANUS TOXOID, AND THE EFFECTS OF SEMINAL PLASMA.

PBMC were cultured for 7 days with tetanus toxoid and with medium alone or 1% SP (v/v).

3.1.3 NK Cell Mediated Cytotoxicity

NK cells are able to lyse certain tumour cell lines and virally infected non-malignant cells without prior immunisation and without MHC restriction. As yet, no unique antigen has been identified on these cells, but the predominant phenotype is CD3 negative, CD4 negative, CD16 (IgG Fc receptor) positive and H-NK positive (Lanier and Phillips 1988). *In vitro* NK cell activity was assessed by measuring cytotoxic activity in a four hour ^{51}chromium release assay (James and Szymaniec 1985). The target cell used was the K562, an MHC deficient tumour cell line derived from a patient with chronic myelogenous leukemia in blast crisis (Lozzio and Lozzio 1975).

Dose response curves.

Various concentrations of seminal plasma were incubated with adherent cell depleted PBMC and target cells for 4 hours at 37°C. Effector:target ratios used were 50:1 and 20:1 (Figure 9). NK cell mediated cytotoxicity as measured by chromium release from target cells decreased with increasing concentrations of seminal plasma. A similar pattern of response was seen at both effector:target ratios.

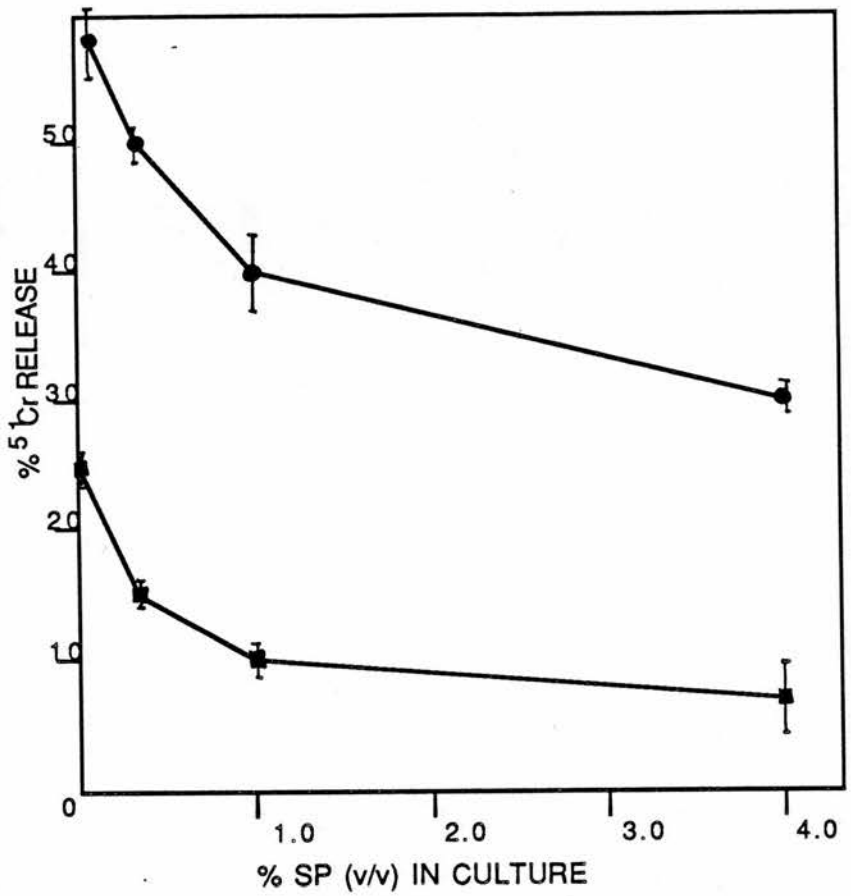

FIG 9 : THE EFFECT OF SEMINAL PLASMA ON NK CELL MEDIATED CYTOTOXICITY.

Adherent cell depleted PBMC were incubated for 4 hours with Cr labelled K562 cells and various concentrations of seminal plasma. Effector : target ratios used were 50:1 (●) and 20:1 (■).(Total release = 2186 cpm)

Preincubation of NK cells.

NK cells were preincubated overnight at 37°C before use, as a previous study in our laboratory had suggested that these cells were less susceptible to suppression (S. Szymaniec, personal communication). In a comparative study with fresh cells (Table 10) it is clear that seminal plasma had very little effect on the preincubated cells, with less than 10% suppression recorded.

Table 10 A.

% SP (v/v) IN CULTURE	% ^{51}Cr RELEASE ± SD	% SUPPRESSION OF CONTROL RESPONSE
0	23.9 ± 2.12	
0.25	14.8 ± 1.96	38.1
4.0	8.2 ± 0.45	65.7

Table 10 B.

% SP (v/v) IN CULTURE	% ^{51}Cr RELEASE ± SD	% SUPPRESSION OF CONTROL RESPONSE
0	21.2 ± 2.3	
0.25	20.3 ± 5.0	4.2
4.0	19.2 ± 6.8	9.3

TABLE 10. A COMPARISON OF THE SUPPRESSIVE EFFECT OF SEMINAL PLASMA ON FRESH AND PREINCUBATED NK CELLS.

Fresh (A) or overnight incubated (B) adherent cell depleted PBMC were incubated for 4 hours with ^{51}Cr labelled K562 cells (Effector target ratio was 25:1 and total release 1600 cpm).

3.1.4 IL-1 Production by Monocytes

IL-1 is normally produced by activated mononuclear phagocytes, common *in vitro* stimuli used include LPS, PMA, whole *Staphylococcus albus*, PHA and Con A. Supernatants are collected between 18 and 48 hours of culture and their IL-1 content is detected by measuring their effect on the proliferation of mouse thymocytes (Gearing *et al* 1985). Stimulation of proliferation is due to IL-2 released by IL-1 stimulated T-cells (Oppenheim and Gery 1982), and the presence of small concentrations of mitogen boosts responses (this is called the costimulator assay - Paetkau *et al.* 1976).

In this study, monocytes were isolated from PBMC, preincubated with or without 1% SP(v/v) for 1 hour, washed and then cultured with or without LPS for 20 hours. This was performed in serum-free medium to reduce background responses. Supernatants were collected and tested in the costimulator assay along with a standard preparation of recombinant IL-1. The full term effect of SP on IL-1 production was not studied as PGE adversely affects the assay (Kristensen *et al.* 1982).

The 48 hour proliferative response of mouse thymocytes to the supernatants is illustrated in Figure 10. Supernatants from LPS stimulated control monocytes induced thymocyte proliferation in a dose-dependent manner. Similar but greater proliferative effects were induced by SP pretreated LPS stimulated monocytes. This difference was highly significant when results were analysed using a paired t-test ($p < 0.02$). Supernatants from unstimulated control and SP pretreated monocytes also induced a proliferative response, but this was very small. Recombinant IL-1 at concentrations between 0.5 to 8.0 U/ml induced a similar measure of proliferative response as the supernatants (Figure 11).

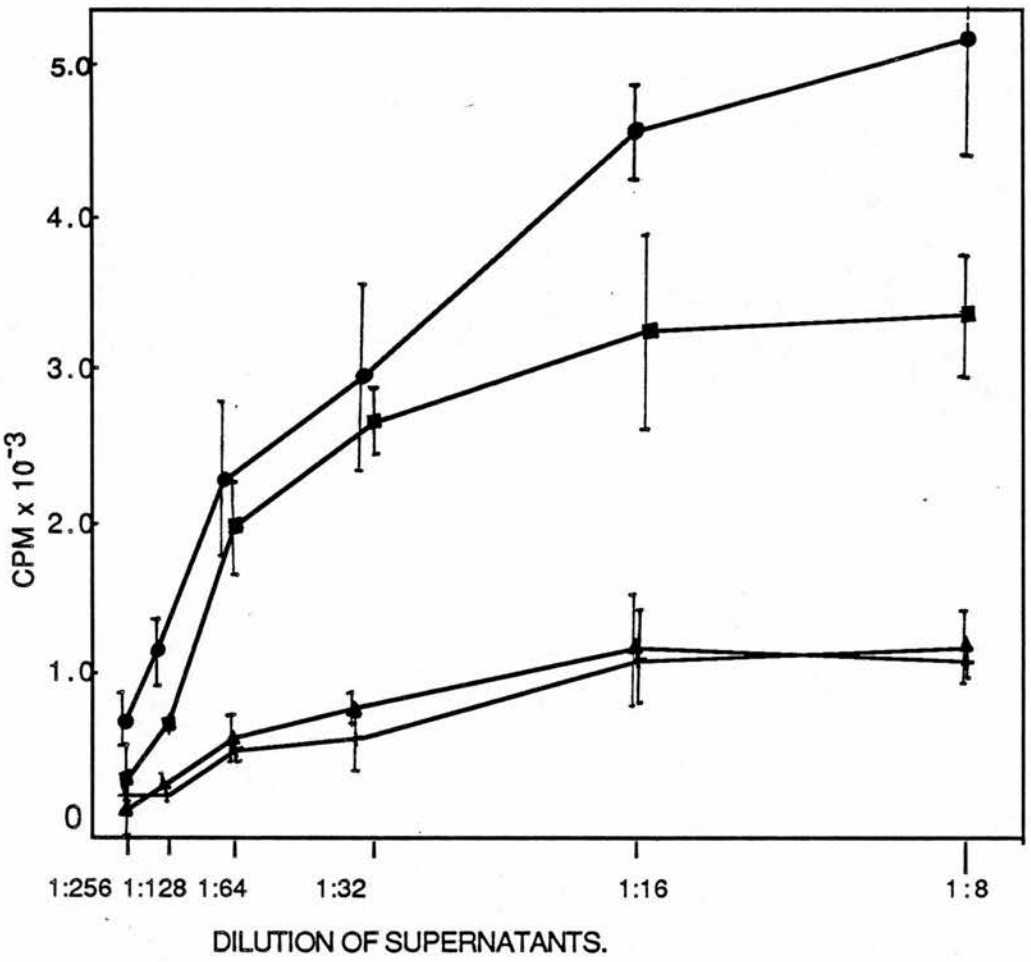

FIG 10 :THE PROLIFERATIVE RESPONSE OF MURINE THYMOCYTES TO SUPERNATANTS FROM LPS STIMULATED MONOCYTES.

Murine thymocytes were cultured for 48 hours with 1 ug/ml PHA and doubling dilutions of supernates from 20 hour cultures of monocytes only(▲), monocytes + 10 ug/ml LPS(■), SP pretreated monocytes only (+) or SP treated monocytes +10 ug/ml LPS(●).

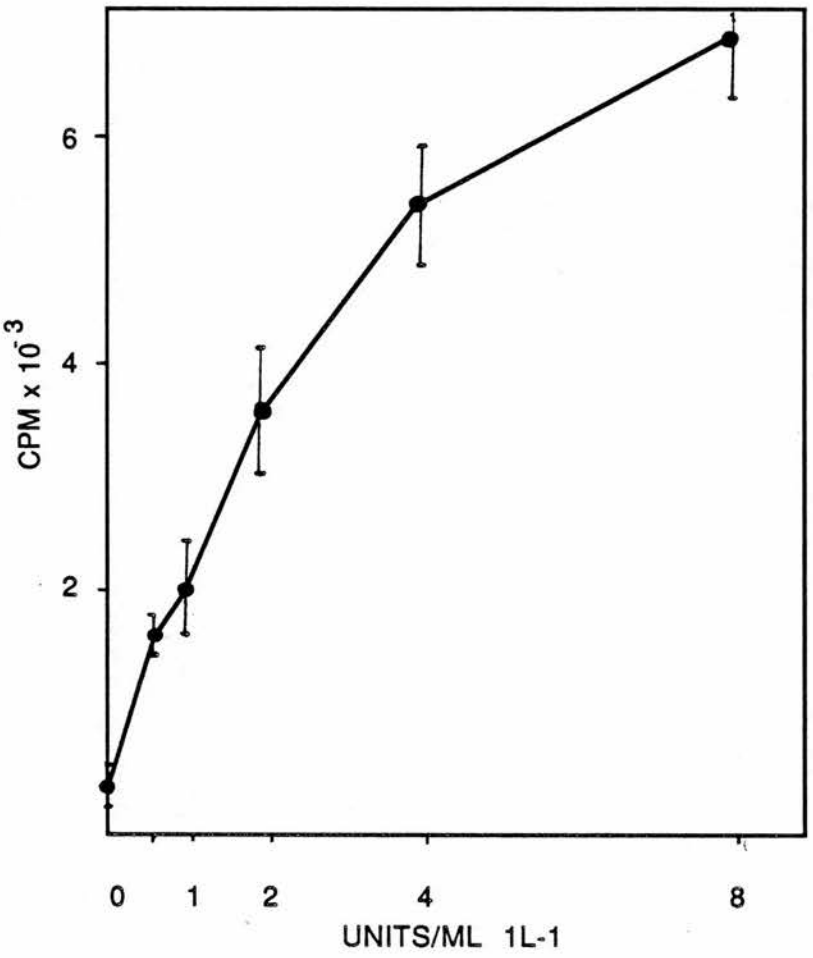

FIG 11 :THE PROLIFERATIVE RESPONSE OF MURINE THYMOCYTES TO RECOMBINANT IL-1

Murine thymocytes were cultured for 48 hours with 1 µg/ml PHA and RPMI only, or doubling dilutions of rIL-1 from 0.5-8.0 Units/ml

3.2 CHARACTERISATION STUDIES

After establishing the major immunological properties of whole seminal plasma, the next phase of the study was to characterise the molecules responsible for the observed suppression. A particular emphasis was made on the prostaglandins of the E series. This was because of their uniquely high concentration in semen, and because initial studies in our laboratory suggested that seminal plasma suppression was mediated by low molecular weight components.

3.2.1 **Dialysis of Seminal Plasma**

Dialysis studies were first performed to establish the molecular weight of possible suppressive components. Seminal plasma was dialysed for 48 hours in dialysis tubing (molecular weight cut off 12 to 14 kD) against 20 volumes of PBS (small volume dialysis) or 2000 volumes of PBS (large volume dialysis). The dialysate from the small volume dialysis was also retained. Whole SP and the three preparations were tested in the lymphoproliferative assay with a sub optimal concentration of PHA, and were analysed for PGE content (Table 11).

The lymphoproliferation results suggest that the greater proportion of suppressive activity is dialysable, but that a significant amount of activity is retained in the SP even after large volume dialysis. Radioimmunoassay for PGE revealed that 94% of the seminal prostaglandins are dialysed out, immediately suggesting that a large molecular weight inhibitor was responsible for the retained inhibition. However, it must be noted that the retained PGE (0.14 µg/ml or 4×10^{-7} M) is an immunologically active concentration).

The whole seminal plasma pools used throughout the study had prostaglandin E levels between 2 to 10 µg/ml.

PREPARATION	PGE µg/ml	+ 2% SP (v/v) OR EQUIVALENT CPM ± SD	% SUPPRESSION OF CONTROL RESPONSE
CONTROL (0 SP)	-	36017 ± 910	
SP	4.34	4605 ± 449	87.3
SP-SVD	0.28	12646 ± 1599	64.9
DIALYSATE	0.18	6931 ± 1461	80.8
SP-LVD	0.14	18679 ± 3731	48.1

TABLE 11. THE DISTRIBUTION OF PROSTAGLANDIN E AND SUPPRESSIVE ACTIVITY IN DIALYSED PREPARATIONS OF SEMINAL PLASMA.

Seminal plasma was dialysed for 48 hours at 4°C against 20 volumes of PBS (SP-SVD) or 2000 volumes of PBS (SP-LVD); the dialysate of the small volume dialysis was also retained. Whole SP and dialysed SP preparations at 2% (v/v) and the dialysate at the equivalent of 2% SP (v/v), were tested in the 90 hour proliferative assay to 5.0 µg/ml PHA.

3.2.2 **Sephadex G200 Gel Filtration**

Seminal plasma was fractionated by gel filtration on a G200 column. Fractions were assayed for protein, and three major peaks identified (Figure 12). The fractions from each peak were pooled, analysed for PGE content and tested in the lymphoproliferation assay at an equivalent concentration to 2% SP (Table 12). PGE was only detected in fraction three, as was suppressive activity. Suppression was not achieved at the very highest concentration of PHA. In this study it was unclear if the prostaglandin which eluted in the third peak was bound to a low molecular weight protein, or was free.

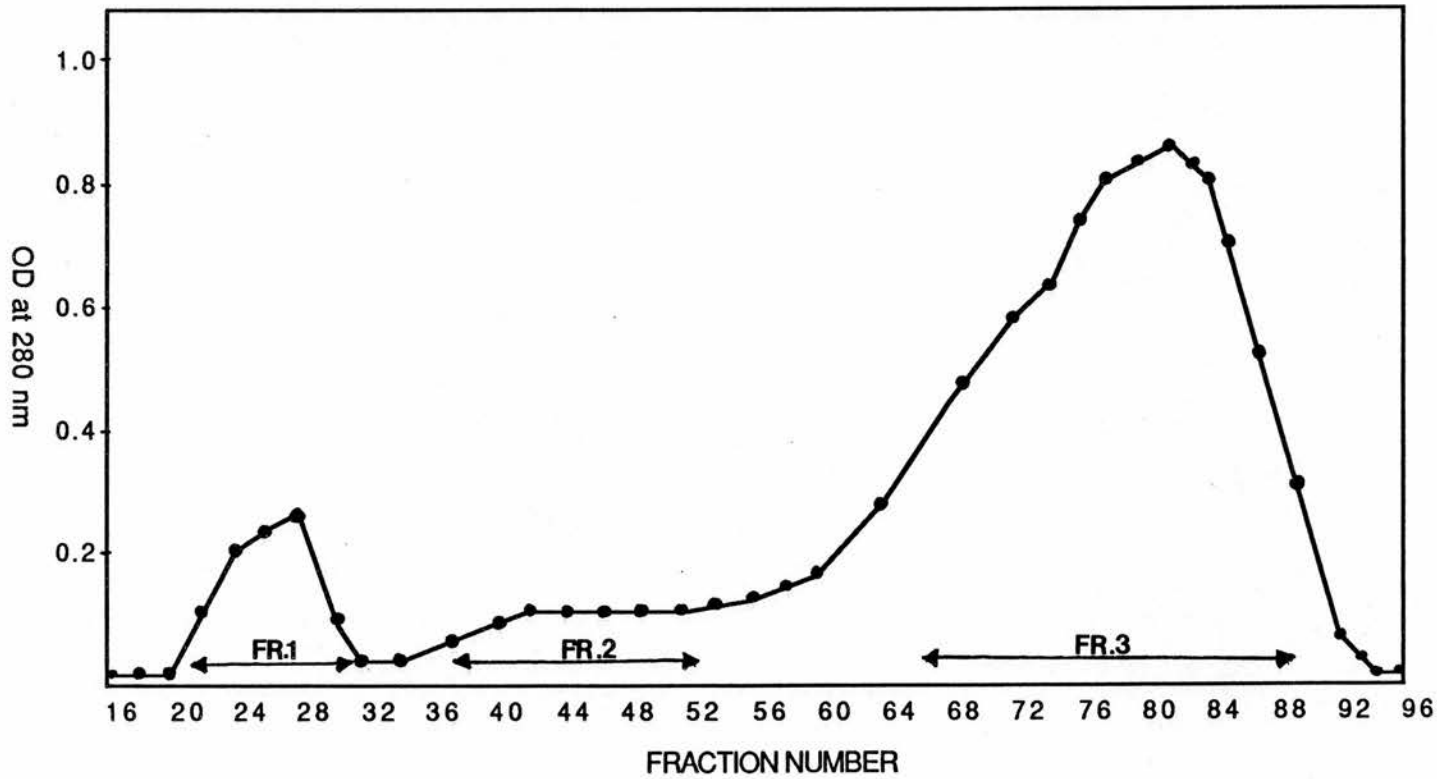

FIG 12 :THE DISTRIBUTION OF PROTEIN IN FRACTIONS OBTAINED FROM A SEPHADEX G200 SEPARATION OF SEMINAL PLASMA

TEST	PGE ng/ml	CPM + SD (% OF CONTROL RESPONSE GIVEN IN BRACKETS) 0 µg/ml PHA	1.25 µg/ml PHA	5.0 µg/ml PHA	15.0 µg/ml PHA
CONTROL		509 ± 48	7766 ± 375	33613 ± 2832	58301 ± 7990
Fr 1	4.3	454 ± 39	9480 ± 324 (122.0)	34043 ± 3709 (101.3)	53847 ± 2462 (92.3)
Fr 2	14.3	674 ± 42	7001 ± 149 (90.2)	39475 ± 4420 (117.4)	60148 ± 1804 (103.3)
Fr 3	222.0	562 ± 96	5261 ± 439 (67.7)	11330 ± 4071 (34.1)	62997 ± 6446 (108.1)

TABLE 12. THE EFFECT OF SEPHADEX G200 FRACTIONS ON LYMPHOPROLIFERATION.

Fractions obtained from a Sephadex G200 separation of seminal plasma were used at an equivalent of 2% SP (v/v) in the 90 hour lymphoproliferative assay to various concentrations of PHA.

3.2.3 Reverse Phase HPLC of Seminal Plasma

Further characterisation of seminal plasma was made by separating a pooled sample by C18 reverse phase chromatography. Fractions were analysed for PGE, 19-OH-PGE, and PGF (Figure 13). PGE was detected in two peaks between fractions 26 and 34 and 38 to 46. A single peak of 19-OH-PGE overlapped the PGE peak between fractions 28 to 34. A very small amount of PGF was distributed between fractions 38 to 45.

Fractions were tested in the NK cell assay (Figure 14 B). Two peaks of suppressive activity were demonstrated between fractions 24 to 35 and fractions 39 to 47. These coincided exactly with the peaks of PGE and 19-OH-PGE.

Fractions were also run in the lymphoproliferation assay using a suboptimal concentration of PHA (Figure 14 C). Components inhibiting proliferation were distributed in two peaks between fractions 24 to 36 and 37 to 52. Again this distribution correlated well with the prostaglandin content of the fractions, except suppressive activity was detected until fraction 52 whilst PGE activity dropped off at fraction 46. This latter suppression may have been due to PGA.

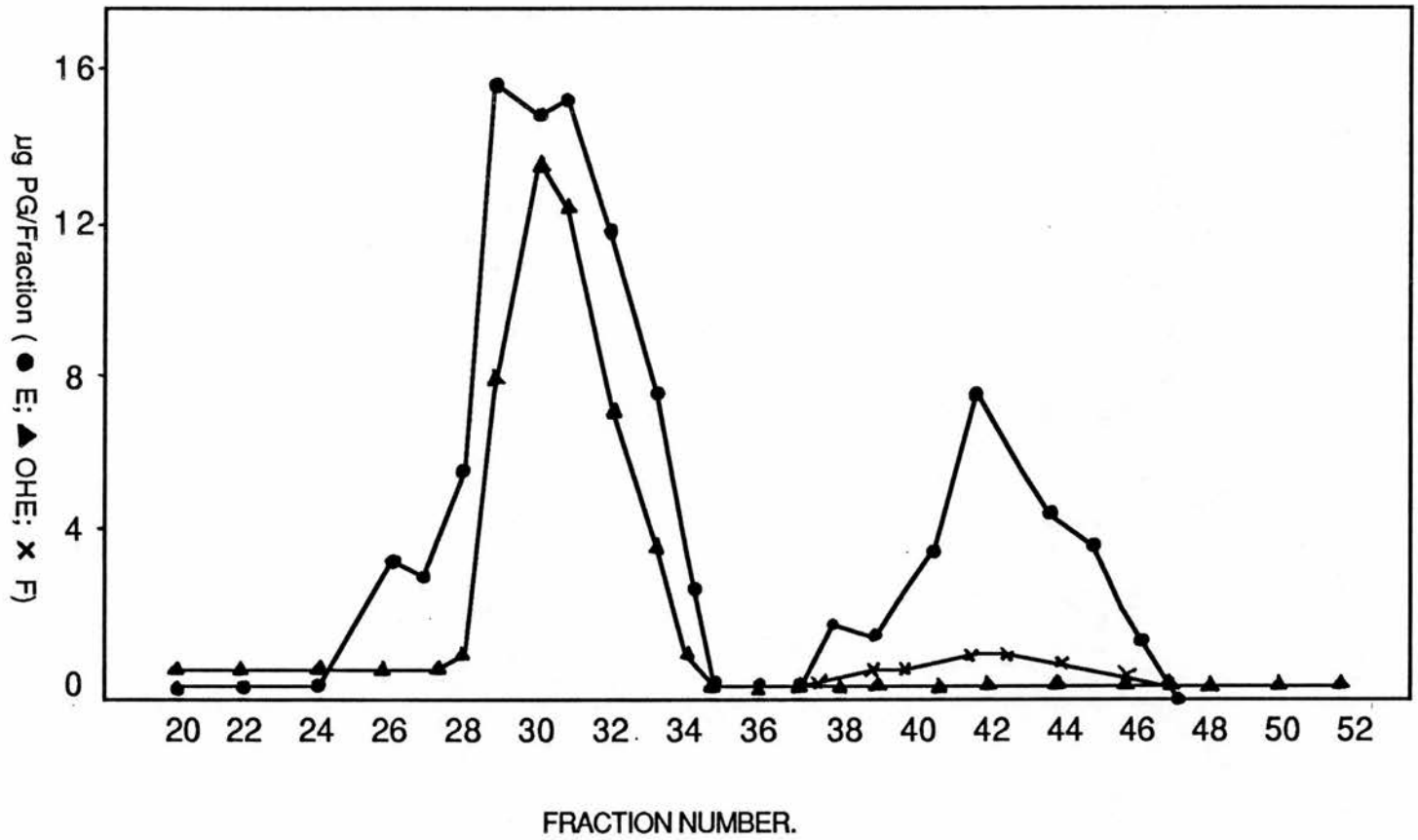

FIG 13: DISTRIBUTION OF PGE 19-0H-PGE AND PGF IN FRACTIONS OBTAINED FROM A REVERSE PHASE HPLC SEPARATION OF SEMINAL PLASMA.

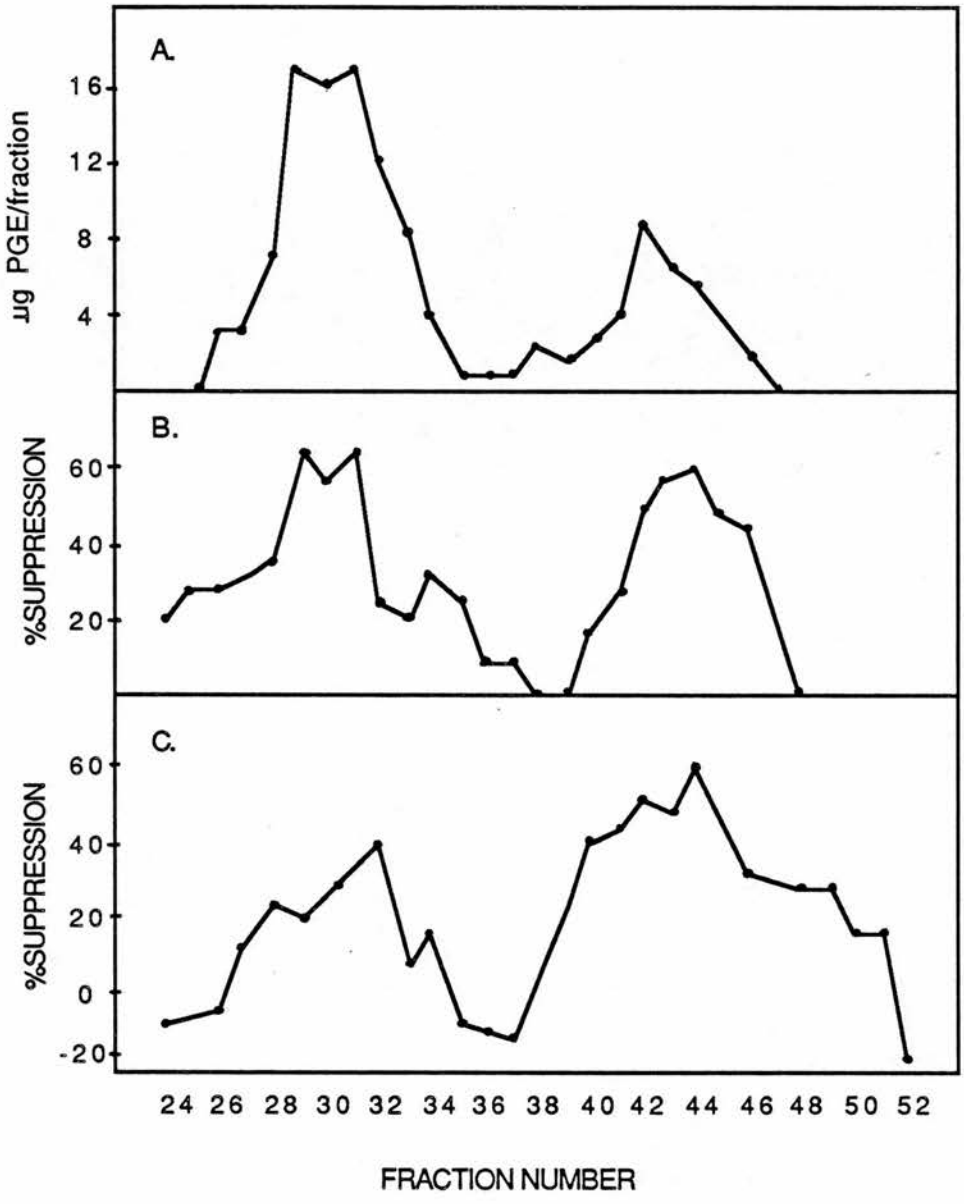

FIG 14 : DISTRIBUTION OF PGE AND IMMUNOSUPPRESSIVE ACTIVITY IN FRACTIONS OBTAINED FROM A REVERSE PHASE HPLC SEPARATION OF SEMINAL PLASMA.

(B:) Adherent cell depleted PBMC were incubated with ^{51}Cr loaded K562, and fractions were used at an equivalent of 2% SP(v/v) in culture. Values are expressed as the % of the control response which was 43.7% ^{51}Cr release. (E:T ratio = 25:1.) Standard deviations are not given, but were <15%

(C:) PBMC were cultured for 90 hours with 5µg/ml PHA and fractions used at an equivalent of 2% SP (v/v) in culture. Values are expressed as a % of the control responsewhich was 33132 ± 3743 (cpm ± sd). Standard deviations are not given, but were <15%.

3.2.4 Delipidation of Seminal Plasma by Absorption Chromatography

Reverse phase HPLC chromatography studies further suggested the involvement of E-series prostaglandins in seminal plasma mediated immunosuppression. The next study therefore involved removal of these molecules and assessing if any inhibitory activity remained. A seminal plasma pool was delipidated and the eluate measured for prostaglandin content and immunological activity.

Delipidation extracted over 99% of the PGE from the seminal plasma sample (the final concentration was 12.4 ng/ml). This procedure also removed two thirds of the SP mediated suppression in the lymphoproliferative assay (Table 13 A), and virtually all the suppressive activity in the NK cell assay (Figure 13 B).

% SP (v/v) IN CULTURE	CPM ± SD	% SUPPRESSION OF CONTROL VALUE
0	22596 ± 3831	
2% SP	4871 ± 761	78.5
2% DLSP	16828 ± 3393	25.6

TABLE 13 A. SUPPRESSION OF PHA INDUCED LYMPHOPROLIFERATION BY SEMINAL PLASMA AND DELIPIDATED SEMINAL PLASMA.

The 90 hour lymphoproliferative response to 1.25 µg/ml PHA was assessed in the presence of 2% SP (v/v) or 2% delipidated (DL) SP (v/v).

%SP (v/v) IN CULTURE	% ^{51}Cr RELEASE	% SUPPRESSION OF CONTROL VALUE
0	24.8 ± 2.2	
0.5% SP	18.9 ± 2.4	23.8
2.0% SP	9.4 ± 1.1	62.1
0.5% DLSP	24.3 ± 0.7	0
2.0% DLSP	22.7 ± 3.2	8.2

TABLE 13 B. SUPPRESSION OF NK CELL MEDIATED CYTOTOXICITY BY SEMINAL PLASMA AND DELIPIDATED SEMINAL PLASMA.

The E:T ratio was 25:1 and total release 3926 cpm.

3.2.5 **Cystic Fibrosis Seminal Plasma Samples**

A natural deficiency of prostaglandins in the ejaculate is recorded in cystic fibrosis patients (Bendvold *et al.* 1986) due to an absence of seminal vesicles (Holsclaw *et al.* 1971).

Samples from three cystic fibrosis patients (courtesy of Dr. Raeburn) were radioimmunoassayed for PGE. Patients 1, 2 and 3 had concentrations of 19.2, 55.3, and 17.9 ng/ml PGE in their seminal fluid compared to 4.34 µg/ml in the normal pool. The suppressive activity of the patient samples were compared with a normal pool in the lymphoproliferative assay to a suboptimal concentration of PHA (1.25 ug/ml) and in the natural killer cell assay (Figure 15).

At a concentration of 1% (v/v) in culture, no differences in suppressive ability of patient samples and the normal pool could be detected in the proliferation assay (Figure 15 A). However, at a lower concentration of SP (0.25%), samples from the cystics were far less inhibitory than the normal pool.

In the NK cell assay, the suppressive ability of patient samples was far less than that of the normal pool at both concentrations used (4% and 1%) (Figure 15 B).

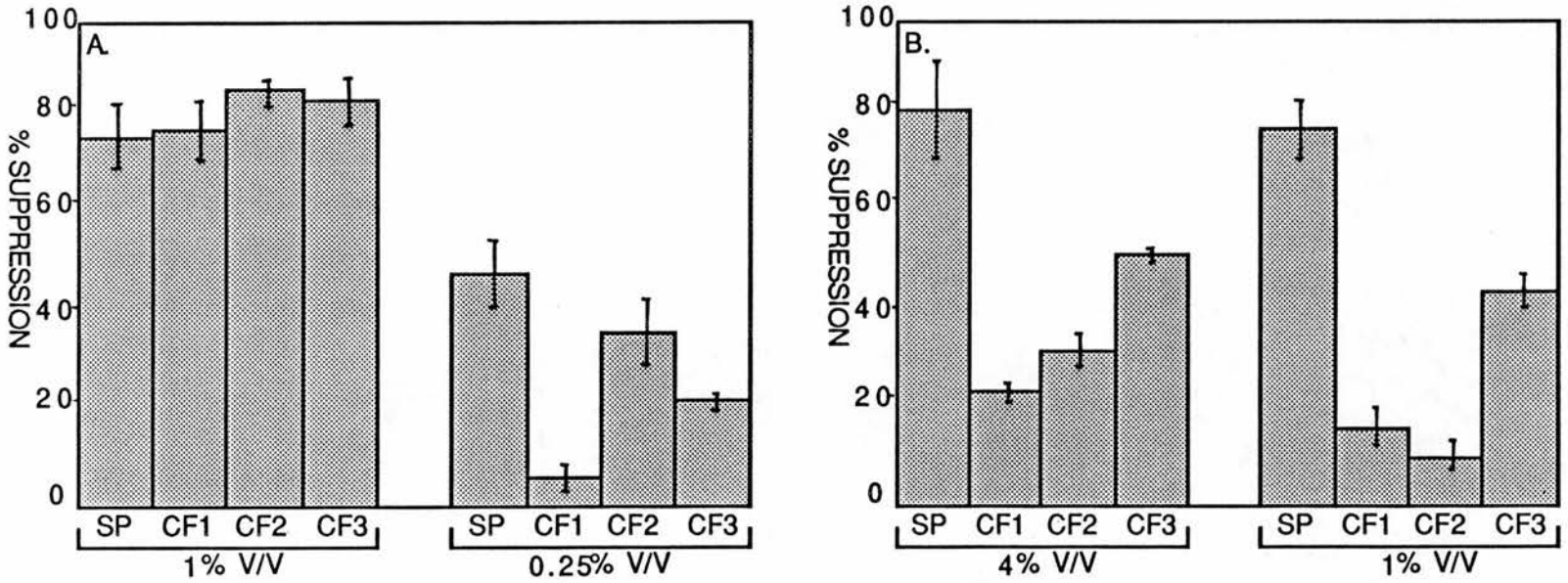

FIG 15 ; A COMPARISON OF THE SUPPRESSIVE EFFECTS ON LYMPHOPROLIFERATION AND NK CELL ACTIVITY OF SEMINAL PLASMA FROM A POOL OF NORMAL MEN AND THREE CYSTIC FIBROSIS PATIENTS.

(A) PBMC were cultured for 90 hours with 1.25µg/PHA and 0.25 or 1% SP(v/v). Results are expressed as a % of the control response without SP which was 22879 ± 2009 (cpm ± sd).
(B) Adherent cell depleted PBMC were incubated with ^{51}Cr loaded K562 cells and 1 or 4 %(v/v) SP. Results are expressed as a% of control response without SP which was 43.7 % ^{51}Cr release E:T ratio = 25:1.

3.2.6 Ion Exchange Chromatography of Seminal Plasma

Early dialysis experiments had suggested that prostaglandins could bind to seminal proteins. To investigate this, a SP pool was desalted by Sephadex gel filtration and the excluded protein was run on a DEAE ion exhange column along with a tritiated PGE tracer. The results confirmed that prostaglandin E bound protein (Figure 16). Two peaks of PGE were located between fractions 2 to 4 and fractions 6 to 12. The PGE tracer eluted between fractions 6 to 10. Analysis of protein content of the fractions revealed a peak in fraction 1 which fell off at fraction 4, and remained at a steady low level to fraction 18.

Fractions were run in the NK cell assay and fractions 2 to 12 were found to have suppressive activity with a peak at fraction 4 and fraction 7 to 12 (Figure 17 B).

In the lymphoproliferative assay to 1.25 µg/ml PHA, fractions 1 to 11 suppressed proliferation, with peak activity occurring in fractions 1 to 4 and 8 to 11 (Figure 17 C).

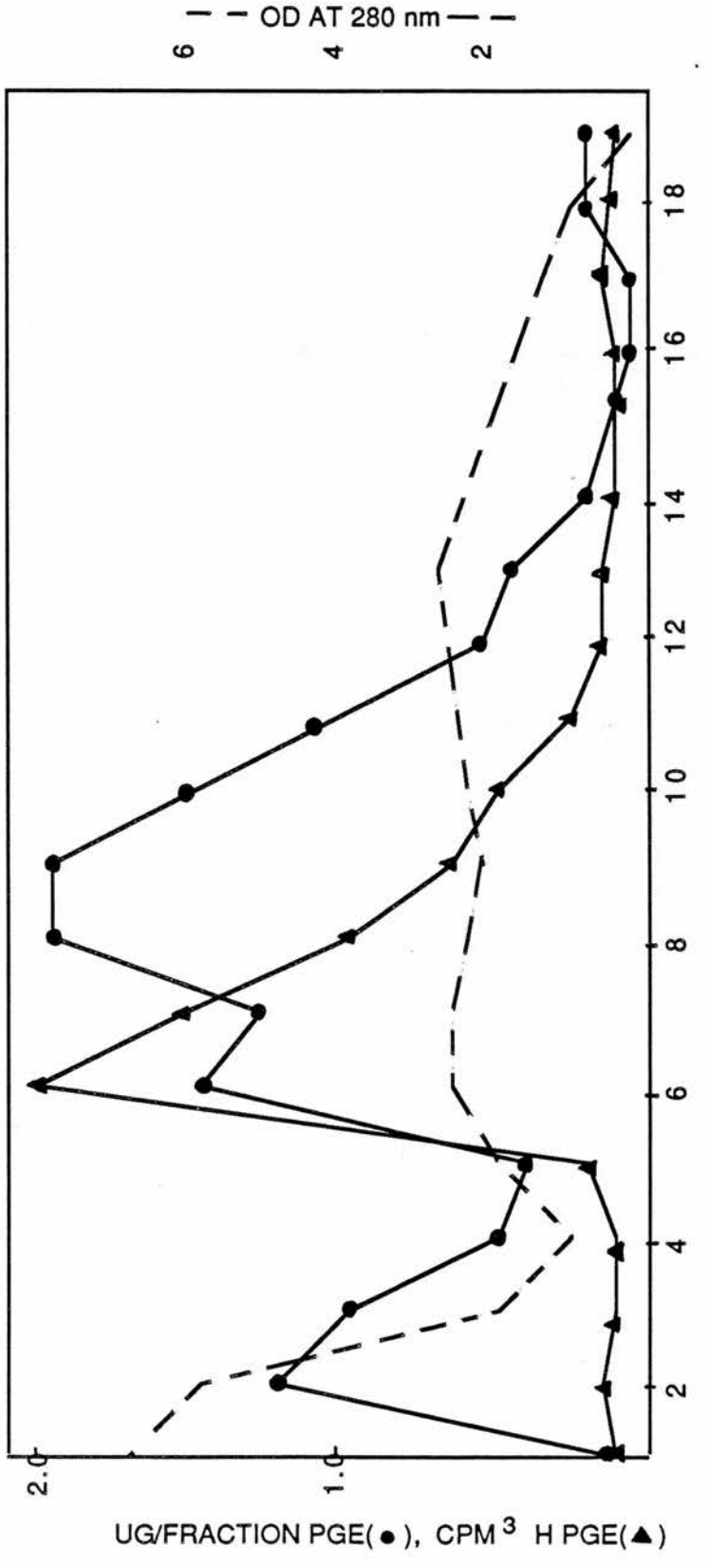

FIG. 16 :THE DISTRIBUTION OF PROTEIN, PGE AND TRITIATED PGE TRACER IN FRACTIONS OBTAINED FROM A DESALTED SEMINAL PLASMA SAMPLE RUN ON A DEAE HPLC COLUMN.

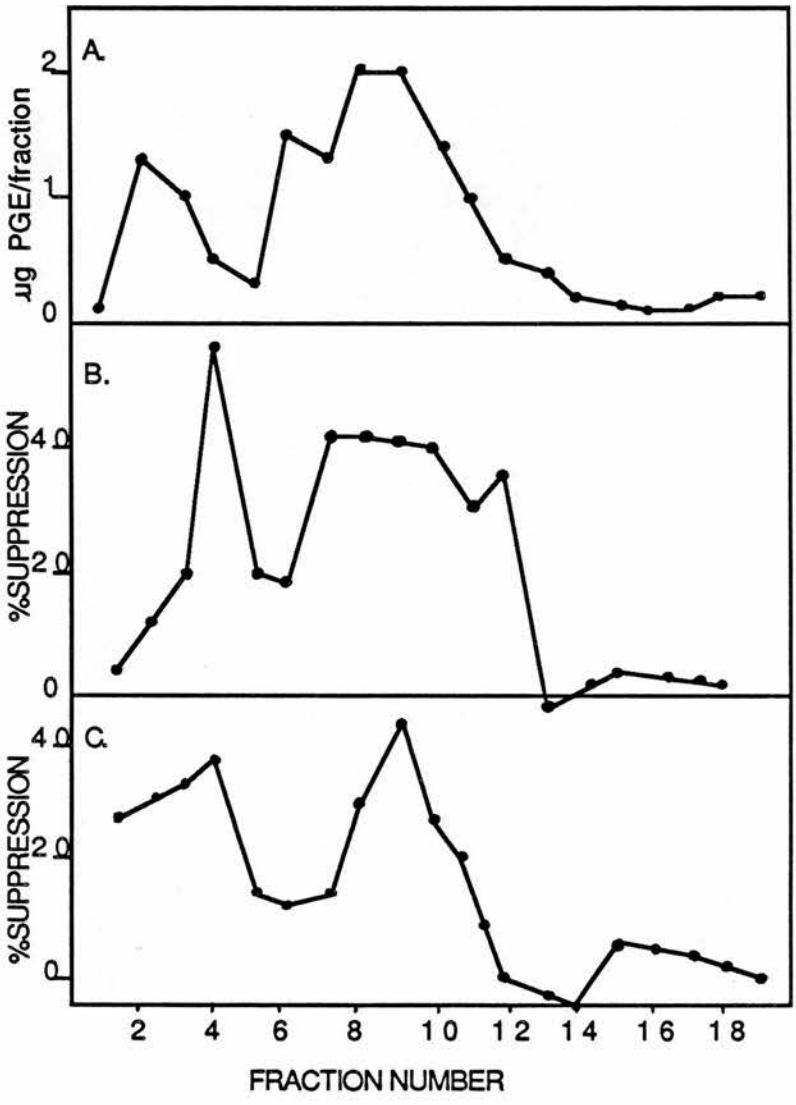

FIG 17 : DISTRIBUTION OF PGE AND IMMUNOSUPPRESSIVE ACTIVITY IN FRACTIONS FROM A DESALTED SEMINAL PLASMA POOL RUN ON A DEAE HPLC COLUMN.

(B.)Adherent cell depleted PBMC were incubated with ^{51}Cr loaded K562 cells and fractions were used at a equivalent of 2%SP(v/v) in culture. Values are expressed as a % of the control response which was 24.8% ^{51}Cr release. (E:T ratio = 25:1.) Standard deviations are not given, but were < 15%.

(C.)PBMC were cultured for 90 hours with 2.5 µg/ml PHA and fractions used at an equivalent of 2% SP (v/v) in culture. Values are expressed as a % of the control response which was 22596 ± 4031 (cpm ± sd). Standard deviations are not given, but were < 15%

3.3 IMMUNOSUPPRESSIVE PROPERTIES OF MAJOR SEMINAL PLASMA COMPONENTS

The complexity of human seminal plasma precluded study of all its components. Characterisation studies had suggested that the inhibitory activity of the secretion was of a low molecular weight, and much of this was contributed by the E-series prostaglandins. In this section, purified preparations of three low molecular weight components of semen with documented immunological properties were studied. These were the prostaglandins, ionic zinc and the polyamine spermidine.

3.3.1 **The Prostaglandins**

The effect of standard prostaglandin preparations on lymphoproliferation.

The 90 hour lymphoproliferative response to 1.25, 5.0 and 20.0 µg/ml PHA was examined in the presence of a wide range (10^{-5} to 10^{-9} M) of PGE_2 concentrations (Figure 18). PGE suppressed proliferation in a dose dependent manner, but these suppressive effects decreased as mitogen concentration increased. The immunological properties of PGE_1 19-OH-PGE_1 and 19-OH-PGF_1 were also compared in the lymphoproliferative assay using a suboptimal (2.5 µg/ml) concentration of PHA (Figure 19). PGE strongly inhibited proliferation at all concentrations tested (10^{-6} to 10^{-9} M), as did 19-OH-PGE at 10^{-6} and 10^{-7} M. At 10^{-8} and 10^{-9} M 19-OH-PGE slightly increased proliferation (< 20%), as did 19-OH-PGF at all concentrations tested (10^{-6} to 10^{-9} M). The small stimulatory effect of 19-OH-PGF (<10%) was not related to its concentration in culture. The average PGE content of seminal plasma samples used throughout the study was approximately 1×10^{-5} M (19-OH-PGE was not analysed).

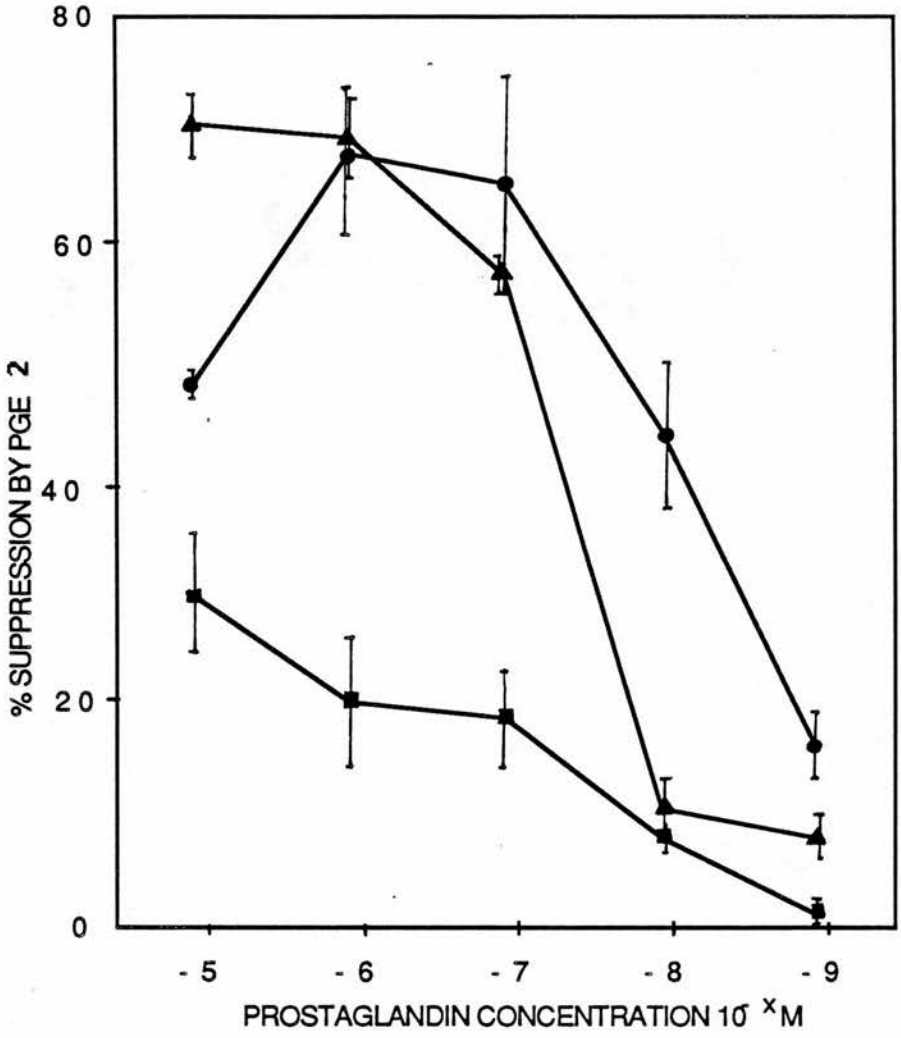

FIG 18 : THE EFFECT OF PGE$_2$ ON LYMPHOPROLIFERATION.

PBMC were cultured for 90 hours with 1.25(●), 5.0(▲) or 20(■)ug/ml PHA and medium alone or 10^{-5} - 10^{-9} M PGE2. Values are expressed as the % suppression of the control culture in the absence of PGE . Control responses in cpm ± sd were 4245 ± 54 at 1.25µg/ml PHA ; 42111 ± 187 at 5µg/ml PHA; and 39330 ± 1373 at 20 µg/ml PHA.

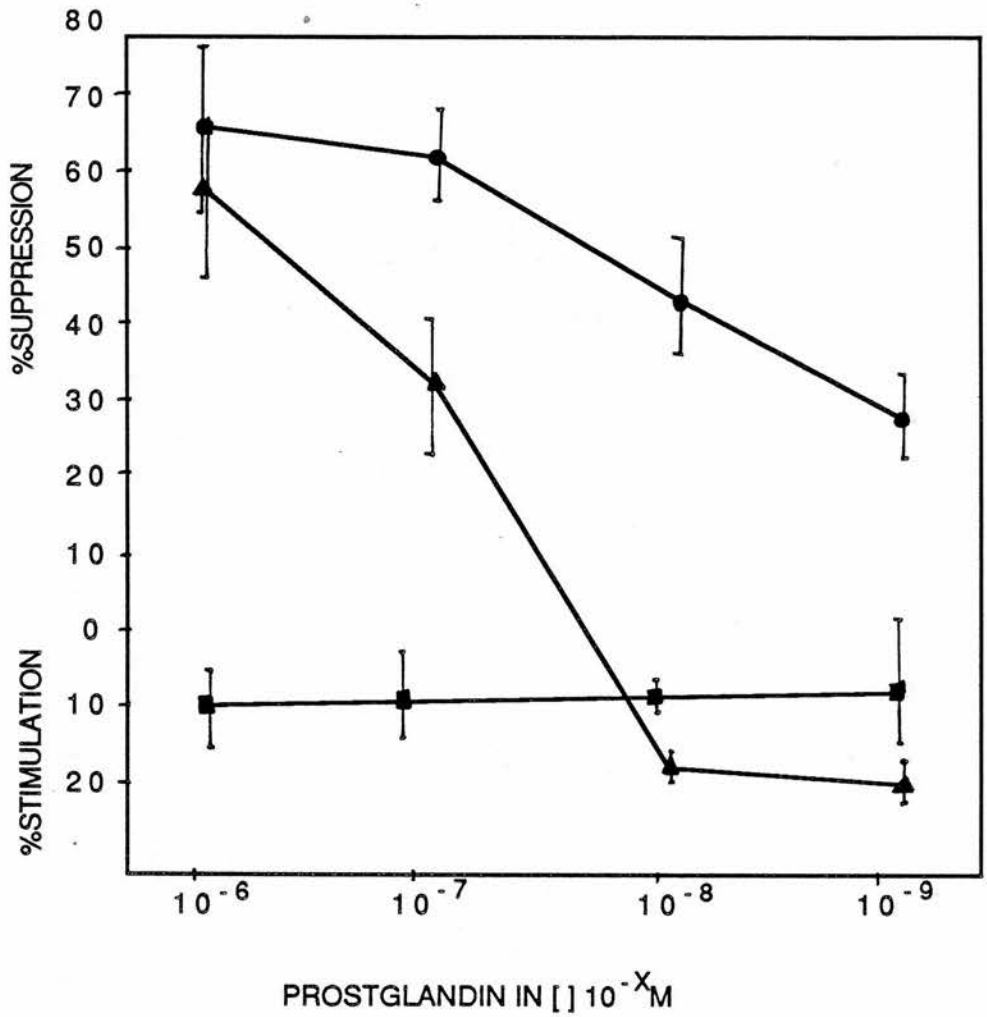

FIG 19 : A COMPARISON OF THE EFFECTS OF PGE_2 ,19-OH-PGE AND 19-OH-PGF ON LYMPHOPROLIFERATION.

PBMC were cultured for 90 hours with 2.5ug/ml PHA and 10^{-6} - 10^{-9} M PGE (●), 19-OH-PGE (▲) or 19-OH-PGF (■). Results are expressed as the % suppression or the % stimulation of the control culture in the absence of PG . This was 23311 ± 491 (cpm± sd).

Reversibility of suppression

Peripheral blood cells were preincubated with 10^{-6} M PGE for 4 hours at 37°C, washed well and then cultured with various concentrations of PHA (Table 14). Results suggest that PGE_2 mediated suppression was fully reversible as proliferative values of prostaglandin pretreated cultures were equal to those of the control cells.

µg/ml PHA	CPM + SD	
	CONTROL	+PGE 10^{-6} M
1.25	8703 ± 765	8408 ± 14
5.0	30600 ± 3360	35137 ± 2862
10.0	28144 ± 3744	26799 ± 1653

TABLE 14. REVERSIBILITY OF PGE_2 MEDIATED SUPPRESSION.

PBMC were preincubated with medium or 10^{-6} M PGE_2 for 4 hours at 37°C, washed x 3 and then cultured for 90 hours with 1.25, 5.0 or 10.0 µg/ml PHA.

Expression of lymphocyte receptors in the presence of prostaglandins

The effect of prostaglandin preparations on induction of the IL-2 and transferrin receptors and possible changes in the CD4 and CD8 positive populations were examined. PBMC were cultured for 60 hours with suboptimal concentrations of PHA, and cells were stained using an indirect immunofluorescent technique, and analysed using the flow cytometer.

PGE_2($10^{-5}$to 10^{-7}M) and seminal plasma (1 and 2% v/v) suppressed the induction of IL-2 and transferrin receptors in a dose dependent manner, and the extent of suppression was similar for both receptors (Table 15).

This decrease in IL-2 receptor expression was accompanied with a decrease in proliferation (Figure 20). Suppression by PGE ($10^{-6}$and 10^{-9} M) and SP (1% v/v) was more effective at 2.5 µg/ml PHA than 10.0 µg/ml; in fact at the higher PHA concentration suppression of receptor expression and proliferation by 10^{-9} M PGE could not be detected.

A comparative study of PGE_2, 19-OH-PGE_1, 19-OH-PGF_1 and SP was made by measuring the induction of IL-2 receptors and number of CD4 and CD8 positive cells in cultures stimulated with a suboptimal (2.5 µg/ml) concentration of PHA (Table 16). PGE was the strongest inhibitor of IL-2 receptor expression. 19-OH-PGE inhibited expression by 10%, but 19-OH-PGF had no effect. The proportion of cells in culture expressing the CD4 and CD8 antigens remained relatively constant in all conditions. The exception was a low CD8 value observed in the PGE treated cells.

TEST	% CELLS POSITIVE (% SUPPRESSION OF CONTROL IN BRACKETS) IL-2 RECEPTOR	TRANSFERRIN-RECEPTOR
UNSTIMULATED CONTROL	4.8	8.1
plus 2 ug/ml PHA:		
CONTROL	44.4	24.2
1% SP (v/v)	14.5 (67.4)	11.2 (53.8)
2% SP (v/v)	8.9 (79.9)	9.4 (61.2)
PGE_2 10^{-5} M	27.8 (37.4)	16.5 (31.9)
PGE_2 10^{-6} M	32.1 (27.7)	20.0 (15.0)
PGE_2 10^{-7} M	38.6 (13.1)	21.2 (12.3)

TABLE 15. EXPRESSION OF IL-2 AND TRANSFERRIN RECEPTORS, AND THE EFFECT OF SEMINAL PLASMA AND PGE_2.

PBMC were cultured for 60 hours with 2.0 ug/ml PHA and seminal plasma or PGE_2. The % of cells in fresh PBMC positive for were IL-2 R = 1.1%, CD4 = 25.2% and CD8 = 21.6%.

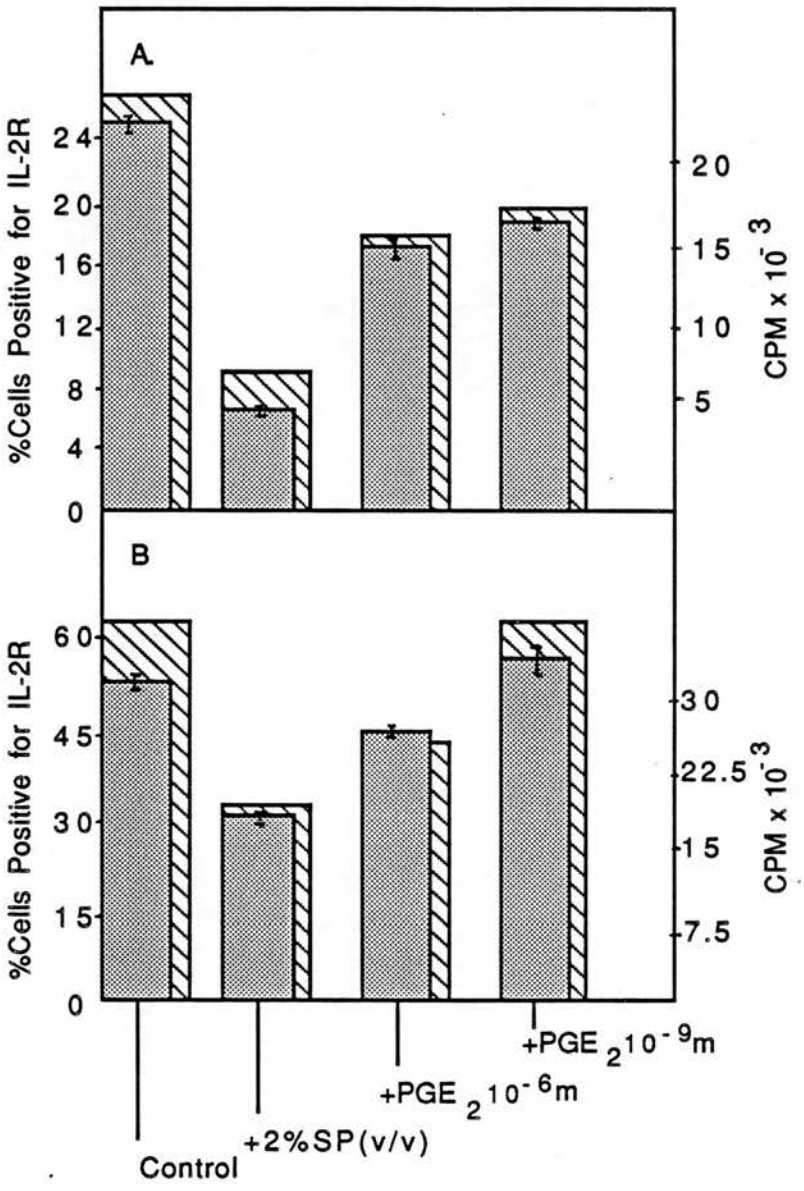

FIG 20 : THE EFFECT OF SEMINAL PLASMA AND PGE_2 ON LYMPHOCYTE PROLIFERATION AND IL-2R EXPRESSION.

PBMC were cultured for 60 hours with 2.5 µg/ml PHA (A.) or 10 µg/ml PHA (B.), and medium alone, 2%SP (v/v) or 10^{-6} or 10^{-9} m PGE. The solid bars represent proliferation values and hatched bars IL-2R expression. The,% positive cells in fresh PBMC were: IL-2R = 1.7% CD4 = 30.1%; CD8 = 11.4 %.

TEST	% CELLS STAINING POSITIVE FOR: IL-2R	CD4	CD8	CD4/CD8 RATIO
CONTROL	25.3	20.8	8.2	2.6
1% SP (v/v)	6.5 (25.7)	26.2	9.2	2.7
$PGE_2 10^{-6}$ M	18.9 (74.7)	21.6	6.8	3.1
19-OH-PGE_1 10^{-6} M	23.0 (90.9)	24.7	10.5	2.4
19-OH-PGF_1 10^{-6} M	25.8 (101.9)	22.0	8.0	2.7

TABLE 16. A COMPARISON OF THE EFFECT OF SEMINAL PLASMA, PGE_2, 19-OH-PGE_1, AND 19-OH-PGF_1 ON IL-2 RECEPTOR CD4, AND CD8 EXPRESSION.

PBMC were cultured for 60 hours with 2.5 µg/ml PHA, and medium alone, 1% SP (v/v) or 10^{-6} M PGE_1, 19-OH-PGE_1 or 19-OH-PGF_1. The bracketed figures are the values expressed as the % suppression of the control receptor expression. The % of cells positive for IL-2R, CD4, and CD8 in fresh PBMC were 3.3, 33.0 and 12.9 respectively.

Addition of recombinant IL-2

PBMC were cultured with PHA (0, 1.25, 5.0 and 10.0 µg/ml), 10^{-6} M PGE_2 and an exogenous IL-2 source (recombinant IL-2 at 2 or 5 U/ml) (Table 17). As reported in an earlier study, IL-2 substantially increased the proliferative values of control cultures with no added mitogen, or with a sub-optimal concentration of mitogen. Addition of 2 and 5 U/ml IL-2 increased the proliferative values of PGE_2 treated cultures to an even greater extent, and no suppression was recorded.

TEST	rIL-2 U/ml	CPM ± SD 0 PHA	1.25 µg/ml PHA	5.0 µg/ml PHA	10.0 µg/ml PHA
CONTROL	0	213 ± 42	2421 ± 558	15047 ± 911	21175 ± 459
PGE_2 10^{-6} M	0	353 ± 58	752 ± 174	4093 ± 658	10490 ± 627
CONTROL	2	443 ± 145	6187 ± 754	15067 ± 83	22491 ± 1763
PGE_2 10^{-6} M	2	547 ± 36	6195 ± 224	12929 ± 311	21566 ± 1506
CONTROL	10	1275 ± 36	7355 ± 1617	16193 ± 940	26262 ± 1740
PGE_2 10^{-6} M	10	1175 ± 53	6879 ± 1052	16823 ± 2346	26566 ± 1506

TABLE 17. THE EFFECT OF RECOMBINANT IL-2 ON PGE_2 INDUCED SUPPRESSION OF LYMPHOPROLIFERATION.

PBMC were cultured for 90 hours with PHA (0, 1.25, 5.0 and 10.0 µg/ml), medium alone or PGE 10^{-6} M and 0, 0.2 or 10 U/ml rIL-2.

The effect of NK cell mediated cytotoxicity

PGE_2, 19-OH-PGE_1 and 19-OH-PGF_1 at concentrations between 10^{-6} and 10^{-9} M and 2% seminal plasma were tested in the NK cell system (Table 18). PGE and 19-OH-PGE suppressed NK cell mediated killing of K562 cells in a similar dose dependent manner. A small decrease (2.5 - 10%) in cytotoxic activity was noted in the presence of 19-OH-PGF.

TEST	NK CELL MEDIATED CYTOTOXICITY	
	% ^{51}Cr RELEASE	% SUPPRESSION
CONTROL	43.7 ± 3.9	
2% SP (v/v)	7.9 ± 1.0	81.9
10^{-6} M PGE_2	17.9 ± 1.2	59.0
10^{-7} M PGE_2	22.2 ± 2.2	49.2
10^{-8} M PGE_2	29.8 ± 1.9	31.8
10^{-9} M PGE_2	38.1 ± 4.1	12.8
10^{-6} M 19-OH-PGE_1	18.9 ± 2.2	56.8
10^{-7} M 19-OH-PGE_1	28.7 ± 2.4	34.3
10^{-8} M 19-OH-PGE_1	27.7 ± 1.2	36.4
10^{-9} M 19-OH-PGE_1	34.4 ± 2.9	21.3
10^{-6} M 19-OH-PGE_1	39.3 ± 3.0	10.1
10^{-7} M 19-OH-PGE_1	39.7 ± 5.1	9.2
10^{-8} M 19-OH-PGE_1	42.4 ± 2.7	3.0
10^{-9} M 19-OH-PGE_1	42.6 ± 2.9	2.5

TABLE 18. THE EFFECT OF SEMINAL PLASMA AND STANDARD PROSTAGLANDIN PREPARATIONS ON NK CELL MEDIATED CYTOTOXICITY.

The E:T ratio was 25:1 and total release 3319 cpm.

3.3.2 Zinc

The effect on the lymphoproliferative response to mitogen

Zinc chloride at 10^{-5} to 10^{-8} M final dilution in culture was examined for effects on the 90 hour proliferative response to 1.25, 5.0 and 20.0 µg/ml PHA (Figure 21). SP at 2% (v/v) was also examined. All concentrations of zinc tested failed to inhibit PHA induced lymphocyte mitogenesis. Basal proliferative values were also unaffected by zinc (Table 19).

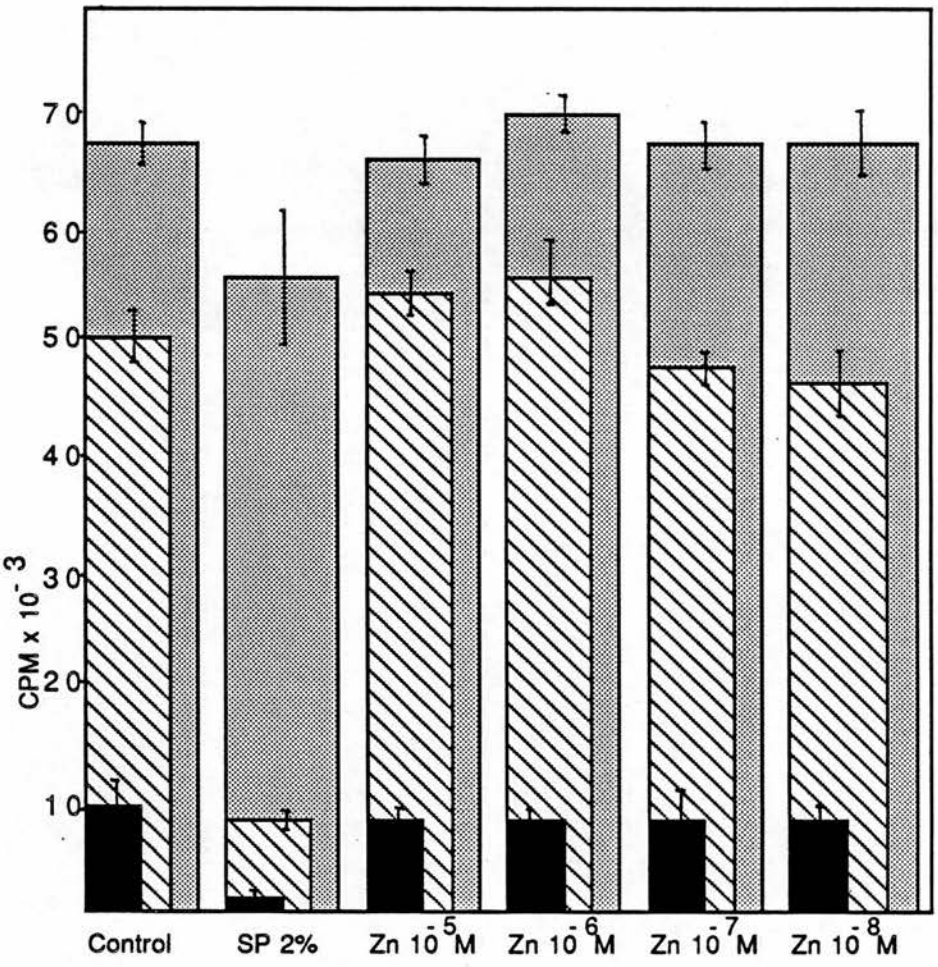

FIG 21 : THE EFFECT OF ZINC ON LYMPHOPROLIFERATION.

PBMC were cultured for 90 hours with 1.25(■), 5.0(▨) or 10.0(▩)µg/ml PHA and medium alone, SP 2%(v/v) or 10^{-5}-10^{-8} M $ZnCl_2$.

ZN CONCENTRATION IN CULTURE	CPM ± SD
0	266 ± 53
10^{-5} M	280 ± 34
10^{-6} M	242 ± 18
10^{-7} M	285 ± 25
10^{-8} M	213 ± 57

TABLE 19. THE EFFECT OF ZINC ON BASAL PROLIFERATION.

PBMC were cultured for 90 hours with various concentrations of zinc.

3.3.3 The Polyamines

Some studies have suggested that in bovine serum supplemented cultures, seminal plasma mediated suppression is due to the generation of cytotoxic molecules following the interaction of bovine serum polyamine oxidase with seminal polyamines (Allen and Roberts 1986, Allen and Roberts 1987, and Vallely *et al.* 1988).

The aim of this section was therefore twofold. Firstly to establish if polyamines could contribute to the reversible and noncytotoxic immunosuppression described using the conditions of this study, that is, in a human serum supplemented culture medium. Secondly, to establish the nature of SP mediated suppression in the bovine serum supplemented system, and to compare this to culture made in RPMI supplemented with human serum, or in serum free medium.

The polyamine used throughout the study was spermidine. Although the concentration of this in semen is ten times less than spermine (0.6 ug/ml), the reported immunological properties are indistinguishable from spermine (Byrd *et al.* 1977) and spermidine was also used in the Vallely study (Vallely *et al.* 1988).

The effect of spermidine and SP on lymphoproliferation and viability

PBMC were cultured for 90 hours with various concentrations of PHA (1.25 - 20.0 µg/ml) and 1% SP (v/v) or 6.0 or 12.0 µg/ml spermidine. Culture medium was RPMI supplemented with 10% human AB serum (Table 20 A) or 10% fetal calf serum (Table 20 B). To assess viability, parallel cultures were made using the same conditions, but without addition of mitogen.

The results in human serum supplemented cultures with SP present were similar to those described earlier; suppression was most effective at sub-optimal PHA concentration, and there was no loss of cell viability. In spermidine containing cultures, suppression was small but variable (0 - 35%), and not related to PHA concentration or loss of cell viability.

In FCS supplemented cultures, SP equally inhibited proliferation induced by all concentrations of PHA. Viability testing indicated this was not because of cytotoxicity. Addition of spermidine to the cultures totally prevented any proliferative response above basal values. Some loss of cell viability was recorded in these cultures.

EXP	TEST	%CELLS VIABLE AT 90 HOURS	CPM + SD (% OF CONTROL RESPONSE IN BRACKETS) 1.25 µg/ml PHA		10 µg/ml PHA (EXP 1&2) 5 µg/ml PHA (EXP 3)		20 µg/ml PHA (EXP 1) 15 µg/ml PHA (EXP 3)	
1.	CONTROL	ND	18619 ± 762		88780 ± 9321		95986 ± 6251	
	1% SP (v/v)	ND	2154 ± 1318	(11.6)	72188 ± 1000	(81.3)	69429 ± 2414	(72.3)
	SPERMIDINE 6 µg/ml	ND	19849 ± 1420	(104.9)	70929 ± 450	(79.9)	79342 ± 3255	(87.5)
2.	CONTROL	97.0	ND		34680 ± 558		ND	
	1% SP (v/v)	89.6	ND		15097 ± 1489	(43.5)	ND	
	SPERMIDINE 6 µg/ml	87.8	ND		33644 ± 320	(97.0)	ND	
3.	CONTROL	99.0	5711 ± 1266		30781 ± 200		29787 ± 22	
	1% SP (v/v)	100.0	1558 ± 179	(27.2)	10731 ± 389	(34.9)	25731 ± 723	(86.4)
	SPERMIDINE 12µg/ml	97.0	3629 ± 392	(63.6)	22900 ± 1267	(74.4)	31599 ± 1206	(106.1)

TABLE 20 A. A SUMMARY OF THREE EXPERIMENTS EXAMINING THE EFFECT OF SPERMIDINE AND SEMINAL PLASMA ON LYMPHOPROLIFERATION IN HUMAN SERUM SUPPLEMENTED RPMI.

PBMC were cultured for 90 hours with medium alone, 1% SP (v/v) or spermidine (6.0 or 12.0 µg/ml) and various concentrations of PHA. Cultures were made in RPMI supplemented with 10% human AB serum.

EXP	TEST	%CELLS VIABLE AT 90 HOURS	CPM + SD (% OF CONTROL RESPONSE IN BRACKETS) 1.25 µg/ml PHA	10 µg/ml PHA (EXP 1&2) 5 µg/ml PHA (EXP 3)	20 µg/ml PHA (EXP 1) 15 µg/ml PHA (EXP 3)
1.	CONTROL	ND	34587 ± 1834	85835 ± 3345	85671 ± 2462
	1% SP (v/v)	ND	8066 ± 1238 (23.2)	17618 ± 10927 (20.5)	12028 ± 5015 (14.0)
	SPERMIDINE 6 µg/ml	ND	235 ± 1420 (0.7)	490 ± 181 (0.6)	445 ± 105 (0.6)
2.	CONTROL	100.0	ND	14873 ± 1824	ND
	1% SP (v/v)	92.9	ND	733 ± 81 (5.0).	ND
	SPERMIDINE 6 µg/ml	61.4	ND	189 ± 14 (1.3)	ND
3.	CONTROL	97.0	5039 ± 377	16360 ± 905	15009 ± 200
	1% SP (v/v)	97.0	1049 ± 225 (20.8)	4910 ± 482 (29.2)	6977 ± 52 (33.2)
	SPERMIDINE 12 µg/ml	78.6	712 ± 189 (14.1)	550 ± 39 (3.4)	782 ± 24 (5.2)

TABLE 20 B. A SUMMARY OF THREE EXPERIMENTS EXAMINING THE EFFECT OF SPERMIDINE AND SEMINAL PLASMA ON LYMPHOPROLIFERATION IN FCS SUPPLEMENTED RPMI.

PBMC were cultured for 90 hours with medium alone, 1% SP (v/v) or spermidine (6.0 or 12.0 µg/ml) and various concentrations of PHA. Cultures were made in RPMI supplemented with 10% FCS.

Reversibility of suppression in FCS and human serum supplemented media

As the previous results had suggested that spermidine treatment could induce toxicity in a proportion of the cell population, preincubation assay was performed to investigate the reversibility of suppression induced by SP and spermidine in the two different media. PBMC were pretreated with 1% SP (v/v) or 12 µg/ml spermidine for 4 hours at 37°C, washed three times and then cultured with various concentrations of PHA for 90 hours (Figure 22).

In AB serum supplemented RPMI, PBMC pretreated with SP and spermidine exhibited similar proliferative values to control cultures, indicating that suppression was completely reversible (Figure 22 A). In contrast, in FCS supplemented cultures, suppression by SP and spermidine was only partially reversible (Figure 22 B).

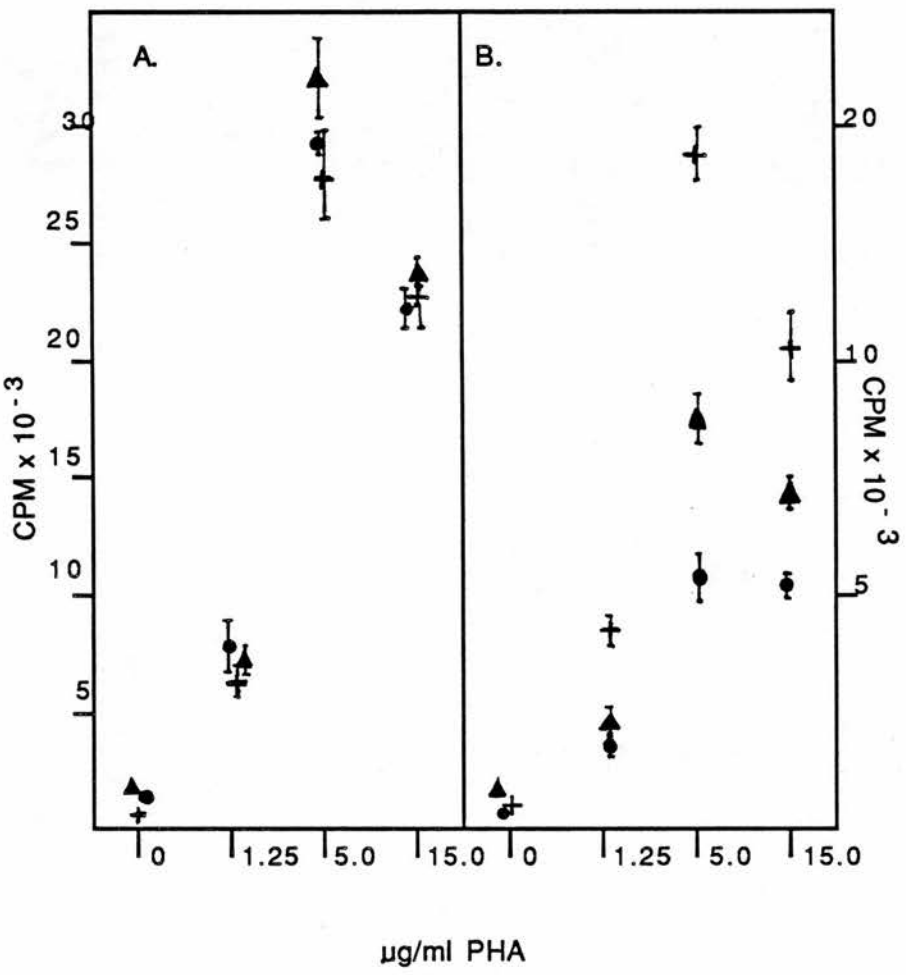

FIG 22 : THE REVERSIBILITY OF SEMINAL PLASMA AND SPERMIDINE MEDIATED SUPPRESSION IN MEDIA SUPPLEMENTED WITH FCS OR AB SERUM.

PBMCwere pretreated for 4 hours at 37 ° C with medium alone (+),1% SP (v/v) (▲) or 12µg/ml. spermidine (●). After washing cells were cultured with PHA for 90 hours. Pretreatment and culture was in AB human serum (A.) or FCS (B.) supplemented RPMI.

A comparison of SP mediated suppression of proliferation in various media

In addition to studies made in human and foetal calf serum, experiments were performed in NBCS supplemented medium as NBCS interaction with SP has been reported to produce greater cytotoxic response than with FCS (Valleley et al. 1988). Studies were also made in serum free medium (HB103) to confirm that components in AB serum were not abrogating the suppressive effects of SP in any way.

Similar patterns of suppression were observed in human serum and in serum free medium (Figure 23 A and B). In summary this was: a lack of inhibition of basal responses, greatest suppression at sub- to optimal PHA concentration, and a decrease in suppression at a post-optimal PHA dose.

Patterns of suppression were also similar in FCS and NBCS (Figure 23 C and D), with a high degree of inhibition of proliferation at all concentrations of PHA. At 90 hours the viability of cells in all four media remained high in the presence of SP (90 - 97%).

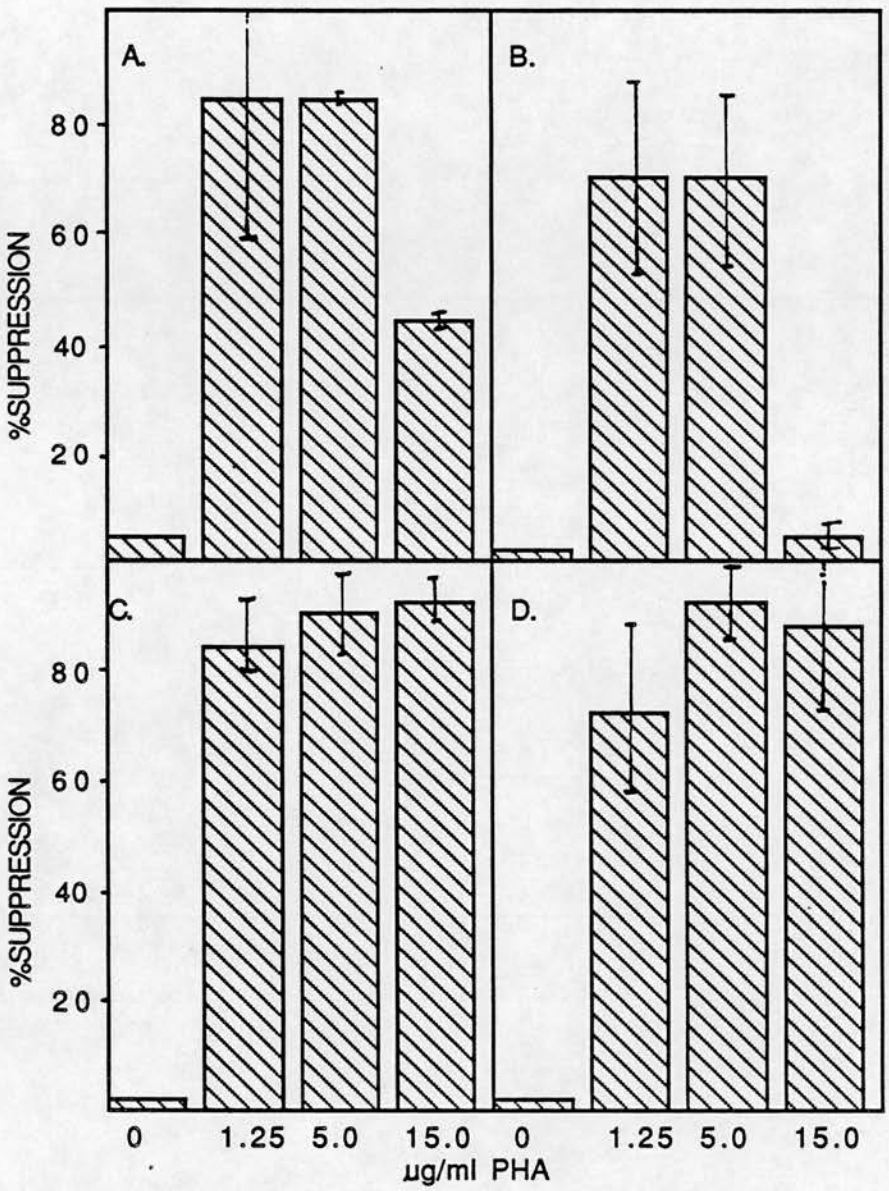

FIG 23 :THE EFFECT OF SEMINAL PLASMA ON LYMPHOPROLIFERATION IN VARIOUS MEDIA.

PBMC were cultured for 90 hours with PHA and medium alone, 2% SP (v/v) in RPMI supplemented with 10% AB serum(A.), FCS(C.) or NBCS,(D.) or in HB103 serum free medium (B.). Values are expressed as a % suppression of the control response without SP. At 1.25, 5 and 10 µg/ml PHA respectively these were : 5711, 30781 & 29787 cpm in AB serum; 3718, 17289 & 16778 cpm in HB103; 5039,16360 &15009 cpm in FCS; and 1802, 7603 & 6323 cpm in NBCS.

NOTE

The majority of the experiments reported here were performed at least three times, and a number of different blood donors were used throughout the study.

CHAPTER 4

DISCUSSION

Seminal plasma is a complex mixture of components, many of which have no ascribed role in sperm function or in the events which lead up to fertilisation. Some of these molecules have known immunological activity, and it is now believed that they may play an important role in preventing an immune response to sperm antigens (James and Hargreave 1984, Alexander and Anderson 1987).

The aim of this study was to make an in-depth examination of the properties of seminal plasma and its components on immune cell function *in vitro*, to contribute to our knowledge on the interaction of the immune and reproductive systems and the biological consequences. The study concentrated on assessing the mechanism of immunosuppression of whole seminal plasma, characterising the components involved, and finally assessing the immunological properties of three of the major low molecular weight components in semen, namely prostaglandins, polyamines and zinc.

All experimental procedures were made in a full human system (seminal plasma, leucocytes and serum supplement), and concentrated primarily on measuring the effects on *in vitro* parameters of T lymphocyte and natural killer cell function.

4.1 IMMUNOLOGICAL PROPERTIES OF WHOLE SEMINAL PLASMA

Immunomodulation of T-cells by whole seminal plasma was assessed primarily by evaluating proliferation and expression of activation markers after stimulation with the T-cell mitogen, phytohaemagglutinin. In three day cultures, immunosuppression was dependent upon the concentration of both SP and PHA. Suppression increased with increasing concentrations of SP, but was most effective at sub-optimal mitogen doses. Basal values were not affected, suggesting SP was inhibiting an event taking place during or after activation of the cells. Lymphocyte viability of SP treated cultures was similar to controls, and reversibility of the suppression could be achieved. These latter observations suggest that SP was not causing suppression of proliferation by cytopathic or cytotoxic mechanisms. The system also statistically confirmed that the pattern of suppression was the same with different pools of seminal plasma and with different lymphocyte donors.

Initiation of a time course study showed that SP most effectively suppressed proliferation when added with, or 4 hours prior to, mitogen. Slightly smaller suppressive values were obtained on addition of SP 24 hours prior to, or 4 hours after, mitogen. Suppression could not be demonstrated when SP was added 24 hours or more after initiation of cultures with mitogen. This indicates that once lymphocytes are activated they become refractory to the effects of SP.

The decrease in suppression observed with increasing concentrations of PHA does question whether components in SP are competitively binding the mitogen. It is known that PHA binding is competitively inhibited by n-acetylglucosamine (Kornfeld and Kornfeld 1971) and n-acetylgalactosamine (Fischer and Mueller 1969), and these are almost certainly found in seminal glycoproteins and polysaccharides. However, similar suppressive activity is noted in antigen stimulated culture, and suppression of other immune cell functions is also noted. In addition, this differential effect of SP in the presence of various concentrations of mitogen is also noted with prostaglandin E, a major constituent of semen (see below).

The clonal expansion of T lymphocytes is critically dependent upon generation of interleukin 2 and its receptors (Cantrell and Smith 1984). Therefore, the next phase of study was to determine their production in seminal plasma supplemented cultures. Results obtained demonstrated that the SP mediated decrease in proliferation was proportionate to the decrease in the number of lymphocytes expressing IL-2 receptors, with suppression most effective in sub-optimally stimulated cultures.

IL-2 production was subsequently measured using a biological (CTLL line) and non-biological (RIA) method of assay. Use of the IL-2 dependent CTLL line was abandoned after SP components were found to directly interfere with CTLL proliferation. The sensitivity of the radioimmunoassay was very poor, and after a number of experiments, a decision was made to discontinue use of this also. Preliminary studies did indicate that SP inhibited IL-2 production by PHA stimulated lymphocytes, and that SP did not interfere with the

radioimmunoassay (data not given) but an improvement in the assay and further experiments would be needed to verify this. The supplementation of cultures with rIL-2 increased proliferation both in sub-optimally stimulated control and SP treated cultures. The proportionate increase was much greater in SP treated cultures, however, and at the highest concentration of rIL-2 used (100 U/ml), proliferative values of 1% SP supplemented cultures equalled those of the controls.

The results suggest that SP components inhibit the T-cell proliferative response to mitogen by impairing IL-2 receptor expression. The time course of IL-2 receptor expression is known to parallel proliferation with both induction and down regulation playing an important role in controlling the response (Cantrell and Smith 1984). However, as IL-2 is also known to amplify expression of IL-2 receptors via effects on the level of mRNA, the primary suppressive effect of SP may be at the level of IL-2 production (Grabstein et al. 1986, Reem et al. 1986).

A number of other laboratories have also studied the basic immunological properties of whole seminal plasma in the human system. Without exception, the inhibitory effects of SP reported are directly related to SP concentration (Stites and Erickson 1975, Lord et al. 1977, Marcus et al. 1987, Majumdar et al. 1982). A decrease in suppressive activity with increased PHA concentrations was also noted (Stites and Erickson 1975, Lord et al. (1977) and Majumdar et al. 1982). Lord et al. (1977) also records similar patterns of response with pokeweed mitogen and concanavalin A. Reversibility of suppression after a one hour preincubation has also been recorded by Majumdar et

al. (1982), and their time course studies record that SP mediated suppression is only achieved when seminal plasma is added with, or 24 hours before mitogen; proliferation values are similar to controls when SP is added 24 or 48 hours after mitogen.

The greatest difference between this study and others made under similar conditions, is the lack of inhibition of the basal response which is noted by the other groups (Stites and Erickson 1974, Lord et al. 1977, and Marcus et al. 1978). This may be explained by differences in the intrinsic stimulatory capacity of different serum supplements used. For example, studies performed in our own laboratory indicate that FCS may contain large amounts of endotoxin, with amounts varying widely from batch to batch. If SP only suppresses cell activational events, use of serum with little stimulatory activity would be unlikely to change basal values, as noted here.

Only one other study has been made on SP mediated suppression in relation to measurement of T-cell growth factors and their receptors. Alexander and Anderson (1987) report that SP does not contain factors which block IL-2 activity. They conclude this is an important observation as IL-2 inhibitors have been found in the decidua during pregnancy, and have been ascribed a role in the maintenance of the foetal allograft (Clark et al. 1984).

Modulation of NK cell activity by whole seminal plasma was assessed by measuring ^{51}chromium release from K562 target cells. Simple studies revealed a dose-response effect of seminal plasma in suppressing NK cell mediated cytotoxicity, but this was abrogated if the effector cells were preincubated for 24 hours at 37°C before use in the assay. This suggests that SP suppresses an early activational event.

Studies made under similar conditions have also demonstrated dose-response effects (Tarter et al. 1986 and Vallely et al. 1988). The inability of SP to suppress preincubated NK cells has also been noted by S. Szymaniac (personal communication). Tarter and coworkers (1986) also found that SP did not affect the lysability of the target cells, and that the suppressive effect of SP was fully reversible.

Assessment of the effects of SP components on monocyte function was made by measuring production of the monokine IL-1. Interleukin 1 is a maturation signal for T-cells, preparing them to respond to antigens or secondary mediator signals (Mizel 1982). Preincubation of monocytes with SP before culture with LPS slightly increased the production of IL-1 above that of control cultures. The effect of addition of seminal plasma into full-term culture with LPS could not be assessed as the prostaglandin in the seminal fluid directly affects mouse thymocyte proliferation (Kristensen et al. 1982). In conclusion, short term exposure of monocytes may slightly stimulate production of IL-1, but the long term effects of SP components on accessory cell function of monocytes remains unknown. The few previous studies reported on the suppression of monocyte function by seminal fluid have measured phagocytosis and oxygen consumption, and have not been made in a full human system (Chvapil et al. 1977 and James et al. 1983).

4.2 THE CONTRIBUTION OF PROSTAGLANDINS TO SEMINAL PLASMA MEDIATED SUPPRESSION

Simple characterisation studies on seminal plasma were made by dialysis. Suppression of T-cell proliferation to PHA was achieved by the dialysed SP and the dialysate, although a larger proportion of the activity was found in the dialysate. Radioimmunoassay revealed that the dialysate was rich in PGE, but a small amount was retained in with the higher molecular weight components. This does indicate that prostaglandin E is a low molecular weight factor contributing to the observed suppression. A G200 fractionation of SP resulted in three peaks of which the third only was associated with suppression of proliferation and with prostaglandin E. As molecular weight marker controls were not used, it is unclear if the prostaglandin was bound to a low molecular weight protein, or whether other low molecular weight components eluted with this fraction.

Seminal plasma was further characterised by reverse phase HPLC, and the peaks of 19-OH-PGE and PGE coincided with the peaks of suppressive activity against NK cell mediated cytotoxicity and the lymphoproliferative response to PHA. Delineation of the relative contribution of the two E-type prostaglandins to suppression was not possible, as the first peak of PGE and the single peak of 19-OH-PGE completely overlapped each other.

Further evidence implicating prostaglandins in the observed suppression of T-cell and NK cell function was made using prostaglandin free seminal plasma. In the first experiment, SP was delipidated, decreasing prostaglandin content of the sample over three hundredfold.

This abrogated all the suppressive activity against NK cell function, and approximately 75% against lymphoproliferation. In a second experiment, prostaglandin free SP samples from cystic fibrosis patients were compared to a normal pool of SP. No differences were found between the pool and the patients SP when used at higher concentrations in the proliferation assay, but when diluted further a difference emerged between the two. In the NK cell assay, suppression by the pool was far greater than the patients at all concentrations tested.

A number of interpretations could be made of these latter findings. Firstly, that prostaglandin E is the major suppressive molecule to NK cell cytotoxicity but that a number of other prostrate derived molecules contribute to the suppression of T-cell proliferation. This interpretation would be in agreement with the conclusions made by Marcus et al. (1987), who could correlate fructose levels with NK cell inhibitory activity but not suppressive activity on PHA induced lymphoproliferation. Secondly, that the apparent differential effects on NK and T-cells may merely reflect differences in the length of the assays. Thus, that prostaglandins only are effective in the shorter NK cell assay, but in the proliferation assay, there is also a contribution from suppressive molecules with a longer time course of action. Thirdly, although the cystic fibrosis data strongly supports the delipidated data that other molecules besides prostaglandins are implicated in T-cell suppression, the relative contribution of prostatic or epididymal components in these cultures may be overestimated. This is because seminal vesicle secretions contribute 50 - 80% of the normal ejaculate.

The final characterisation study was initiated to investigate the possibility that prostaglandins bound to seminal proteins in the ejaculate. Desalting of a SP sample and separation of the excluded proteins showed that two peaks of PGE activity were retained, and these correlated well with suppression of proliferation and NK cell mediated cytotoxicity. This confirms that the E-series prostaglandins can bind seminal proteins, and that these bound prostaglandins retain immunological activity. Whether this is due to release of the prostaglandin in culture or reflects activity of the complex remains to be established. It also remains to be established if previously described high molecular weight suppressive proteins are in fact protein prostaglandin complexes (Prakash et al. 1976, Lord et al. 1977).

To compare the mechanisms of action of whole seminal plasma with pure prostaglandin preparations, a series of proliferation and NK assays were run with PGE_2, 19-OH-PGE_1 and 19-OH-F_1. PGE_2 (10^{-5} to 10^{-9} M) and 19-OH-PGE_1 (10^{-5} to 10^{-6} M) suppressed proliferation, and were most effective at a suboptimal stimulatory dose of mitogen. Suppression of proliferation was accompanied by a decreased number of cells expressing receptors for IL-2 and transferrin. 19-OH-F_1 had no effect on proliferation or receptor expression. PGE_2 and mediated suppression was reversible and could be overcome by addition of recombinant IL-2. PGE_2 and 19-OH-PGE_1 (10^{-5} to 10^{-9} M) also inhibited NK cell mediated cytotoxicity in a dose dependent manner, but 19-OH-F_1 had only a small amount of inhibitory activity (< 10%). These results confirm that the E series of prostaglandins are important

suppressors of immune cell function and that their mechanism of action is very similar to that of whole seminal plasma.

Despite the high concentration of prostaglandins in semen and the known immunoregulatory properties of PGE, only two other characterisation studies have been made with respect to these molecules. In 1986 Tarter *et al.* identified 19-OH-PGE_1 and $_2$ rich fractions from a HPLC separation of the acid lipid fraction of semen as the major suppressive molecules against NK cell activity. In 1988, Vallely *et al.* demonstrated that a PGE_2 rich fraction from a separation of seminal plasma by ion exchange chromatography also caused suppression of NK cell cytotoxicity under certain conditions. The contribution of prostaglandin E in Tarter's system and 19-OH-PGE in Vallely's system is unclear, and no further studies have been reported by either laboratory.

The differentiation and proliferation of all leucocyte types is regulated by a complex network of growth factors and hormones, and evidence over the last 18 years establishes prostaglandin E as an important participant in these processes (reviewed by Goodwin and Ceuppens 1983, Wickremasinghe 1987).

The mechanism of action of PGE in controlling lymphocyte function is by raising intracellular cyclic AMP (Makoul *et al.* 1985). This elevation is mediated via activation of adenylate cyclase which is coupled to cell surface receptors via G proteins (Nishizuka 1986). Increased cyclic AMP levels in lymphocytes are associated with decreased proliferation (Goodwin *et al.* 1979), production of IL-2

(Chouaib *et al.* 1985), and expression of transferrin receptors (Chouaib *et al.* 1985).

Evidence is emerging that control of lymphocyte responses may be through the convergence of prostaglandin E and interleukin 2 on the adenylate cyclase system. Interleukin 2 is a positive signal for lymphocyte differentiation and proliferation, and binding of this growth factor to its receptor promotes translocation of protein kinase C to the plasma membrane and the phosphorylation of membrane substrates including the IL-2 receptor (Farrar and Anderson 1985, Farrar and Taguchi 1985). Basal adenylate cyclase activity and PGE_2 stimulated cAMP accumulation are also decreased proportionately to increased concentrations of exogenous IL-2 (Beckner and Farrar 1986). Membrane studies indicate that IL-2 effects on the adenylate cyclase system are indirect and mediated by protein kinase C in contrast to PGE_2 which directly affects adenylate cyclase (Beckner and Farrar 1986).

In conclusion, there appears to be a potential bidirectional modulation of lymphocyte proliferative response under the regulation of prostaglandin E and interleukin 2, which converges at the adenylate cyclase system. This supports the data presented here in that E series prostaglandins decreased T-cell proliferation, IL-2 production and IL-2 and transferrin receptor expression. As previously stated, IL-2 amplifies expression of IL-2 receptors, and similar findings have been made on expression of transferrin receptors (Pelosi-Testa *et al.* 1988). This control system is probably important *in vivo*, as a mechanism of modulating biological responsiveness.

Suppression of NK cell mediated cytotoxicity by PGE is also mediated by raising intracellular cyclic AMP (Goto et al. 1983). Activation of NK cells can be achieved with interleukin 2 (Trinchiera et al. 1984, Seki et al. 1985) and interferon (Koren and Leung 1982). It is therefore possible that control of NK cell function could also be made by IL-2 and PGE by a convergence of the adenylate cyclase system.

Monocyte macrophage function is also reported to be modulated by prostaglandin E. PGE_2 can suppress Ia expression and interleukin 1 production, whereas indomethacin augments these two functions (Snyder et al. 1982 and Kunkel et al. 1986). The mechanism of action of PGE in modulating Ia expression is through raising intracellular cAMP (Snyder et al. 1982). The effect of PGE on monocytes was not investigated in this study, but other workers in this laboratory have found human monocytes and rat macrophages relatively refractory to short and long term exposure to PGE_2 and SP when measuring superoxide anion production (K. Donaldson and S. Szymaniec personal communication). These findings therefore warrant further investigation.

The mechanism of action of 19-OH-PGE is unknown, although it is likely to be similar to PGE (Kelly, personal communication).

4.3 THE CONTRIBUTION OF ZINC TO SEMINAL PLASMA MEDIATED SUPPRESSION

Lymphoproliferative responses to PHA were not affected by zinc concentrations similar to those found in seminal plasma. No other studies on the contribution of zinc to SP mediated suppression of lymphoproliferation have been made. Zinc rich fractions from human prostatic fluid are however, reported as suppressive to murine peritoneal macrophage function (Chvapil et al. 1977).

Zinc is essential for lymphocyte proliferation and for production of lymphokines (Chesters 1972). It is also reported as mitogenic to murine lymphocytes, but suppressive in the presence of PHA (Berger and Skinner 1974). A report also suggests that murine NK cell function is inhibited (Ferry and Donner 1984). The discrepancy between results obtained here and those previously reported are unexplainable, unless there is a differential effect of zinc on murine and human leucocytes.

4.4 THE CONTRIBUTION OF POLYAMINES TO SEMINAL PLASMA MEDIATED SUPPRESSION

These studies help to clarify the question as to whether reported SP mediated suppression is merely an artefact created by interaction of bovine serum and seminal polyamines. It is clear from the human serum and serum free studies that suppression of immune cell function occurs outside a bovine supplemented system. This is supported by other studies made in human serum supplemented cultures (Stites and Erickson 1975, Lord *et al.* 1977, Marcus *et al.* 1978, Majumdar *et al.* 1982). Some of this may be due to spermidine itself. This has not been reported in *in vitro* assays by others, but Shohat *et al.* (1988) report that low levels of spermine and spermidine in seminal plasmal was related to infertility, the presence of antisperm antibodies and lack of immunosuppressive activity.

The results do however suggest that in bovine serum supplemented cultures, cytotoxic or cytopathic factors are generated as a result of the interaction between serum polyamine oxidase and spermidine. Using pure spermidine, no loss of cell viability was recorded before 48 hours but at 90 hours a proportion of cells were killed (data not shown). Toxicity was not demonstrated with seminal plasma, but the inability of cells to completely recover after treatment also suggested the generation of cytopathic molecules not present in a bovine serum free system.

A number of studies have been undertaken to ascertain the result of polyamine oxidase and polyamine interactions on cell function and

variable results have been reported. Byrd, Jacobs and Amoss (1977) reported that synthetic polyamines were not themselves inhibitory to lymphoproliferation in human serum supplemented cultures, but were converted to active moieties in the presence of foetal calf serum. This inhibitory activity was not due to cytotoxicity and was fully reversible. In contrast, Tabor *et al.* (1964) found that a specific amine oxidase of ruminant sera could convert spermine and spermidine into aminoaldehydes which were toxic to cells in culture. Allen and Roberts (1987) and Vallely *et al.* (1986) also identified loss of cell viability after 24 hours in culture, associated with bovine serum interaction with spermine, spermidine and seminal plasma.

In conclusion, the combined presence of bovine serum and seminal plasma leads to the generation of products which are cytotoxic or cytopathic to cells in culture, depending on the culture system. This is an entirely separate mechanism of cell suppression by seminal plasma to the suppression generated in a human serum or serum free system. Reports of immunosuppression by SP in FCS and NBCS containing systems must, therefore, be treated with caution.

As a final note, semen itself does contain an intrinsic oxidase activity and if left to stand molecules causing cytopathic or cytotoxic effects could possibly be generated (Williams-Ashman, personal communication). Although toxicity was not recorded here, generation of cytopathic molecules could not be ruled out of this study, and may also be a relevant suppressive mechanism *in vivo*.

4.5 THE CONTRIBUTION OF OTHER SEMINAL COMPONENTS TO SUPPRESSION

Many of the other components of seminal plasma have ascribed immunological activity besides the prostaglandins, polyamines and zinc, and these cannot be excluded from a possible role in seminal plasma mediated immunosuppression. They may be of particular importance in suppression of the function of immune cells not looked at here, or their activity may have a greater relative importance in a different species.

4.6 THE *IN VIVO* RELEVANCE OF *IN VITRO* SEMINAL PLASMA MEDIATED SUPPRESSION

Under normal circumstances, autoimmune responses to sperm antigens are not detectable in the male. Early studies led to the belief that the blood-testis barrier was absolute, preventing any contact of developing spermatozoa with cells of the immune system. It is now clear, however, that in some areas there is leakage of sperm antigens (Tung *et al.* 1988) and active local suppression by CD8 positive cells may prevent sensitisation (El Demiry *et al.* 1987). It is inconceivable that factors in the seminal vesicles and prostate could participate in local suppressive mechanisms, but epididymal derived suppressive molecules have been demonstrated, and these could constitute an immunological back up to suppressor cells (Anderson and Tarter 1982).

Alloimmune responses to spermatozoa do not usually occur in the female, even though the vaginal route is adequate for immunisation against a variety of antigens (Straus 1961, Yang and Schumaker 1979). Furthermore, in the rabbit a vaginal leucocytosis in induced upon insemination and phagocytosis of spermatozoa has been demonstrated (Austin 1976, Phillips and Mahler 1975). Insemination of a second ejaculate into a leucocyte dominated vaginal lumen showed no loss of fecundicity, suggesting that the phagocytic function was to remove the pool of non-fertile sperm, and possibly to 'select' fertile sperm (Taylor *et al.* 1982). The situation in the human is not so clear; Pandya and Cohen (1985) have observed a leucocytosis in the cervix of females after artificial insemination, but Wolff has not recorded this

phenomenon after natural coitus (H. Wolff, personal communication). If a leucocytosis does occur and polymorphs and macrophages do degrade 'unwanted' sperm, it does raise the question to the *in vivo* relevance of *in vitro* seminal plasma suppression. However, studies in our laboratory have revealed that macrophages are relatively resistant to the suppressive effects of seminal plasma and prostaglandin E *in vitro*, indicating that there may be a differential effect on cells of the immune response. Hence macrophages could degrade spermatozoa and debris from the insemination site, but under similar conditions the function of lymphocytes remained paralysed. The *in vivo* concentration of prostaglandins at an insemination site remains unknown, but 19-OH-PGE and 19-OH-PGF can still be detected in the human vagina up to 72 hours after intercourse (Sutton *et al.* 1987).

4.7 THE CLINICAL IMPLICATIONS OF SEMINAL PLASMA MEDIATED SUPPRESSION

If the primary role of seminal plasma is to protect immunologically foreign sperm from immune recognition, it follows that certain categories of infertility may be caused by a deficiency of key suppressive factors in the semen. This has been demonstrated with two of the seminal components studied in this thesis: the polyamines and the prostaglandins. Decreased seminal spermine and spermidine levels have been associated with infertility, antisperm antibodies and decreased *in vitro* immunosuppression (Shohat *et al.* 1988). Low levels of prostaglandin E are found in patients who are diagnosed with unexplained infertility (Bygdeman *et al.* 1970).

A number of possible immunological sequela could result from a deficiency of seminal suppressive components. Firstly, if leucocytes present in ejaculate derive from sites of infection they will be preactivated, but paralysed in function due to the high concentration of suppressive factors present in the semen. If these factors are missing the activated cells could initiate a graft-versus-host response in the female tract, or secreted products of activation could cause spermatozoal damage. A recent study has shown that interferon and tumor necrosis factor have an adverse affect on sperm motility *in vitro* (Hill *et al.* 1987). Additionally, patients with greater than one million/ml leucocytes were reported to have significantly reduced semen parameters compared to those who had less than one million/ml cells (Wolff and Anderson, 1988b). Secondly, suppressive factors secreted by, for example, the epididymis, could

provide a 'back up' mechanism to prevent sensitisation of the male to his own spermatozoa, and a deficiency may lead to production of antisperm antibodies. Thirdly, a decreased suppressive activity of SP would allow sensitisation in the female tract, possibly resulting in production of antisperm antibodies or killing of spermatozoa by activated cells, each of which would result in decreased fertility.

A negative effect of a local immunosuppression in the male and female reproductive tract may also be the protection of micro-organisms from immune destruction. Impairment of the immune response could for example, prevent recognition and destruction by antibodies, cytotoxic T-cells, NK cells, and phagocytosis and killing by polymorphs and macrophages. *In vitro* impairment of these functions has been demonstrated repeatedly, and if these findings are relevant *in vivo*, then continuous exposure to seminal plasma would aid transmission of and impair the host response to these organisms.

The increasing world incidence of AIDS makes an understanding of transmission of HIV of particular concern. The virus has been isolated from seminal leucocytes (Zagury *et al.* 1984) and is found free in semen (Ho *et al.* 1984). Initially, a high profile disease in homosexuals, epidemiological study reveals that passive anal genital intercourse is the lifestyle factor with the highest risk of HIV infection (Marmor 1985). Measurement of PGE metabolites in the blood of rhesus macaque monkeys after rectal insemination demonstrates that absorption of SP components can occur (Alexander *et al.* 1986), and similar studies in rabbits shows an impairment in the systemic

immune response (Richards et al. 1984). It is also known from in vitro study that prostaglandin E increases HIV shedding from an infected T-cell line (Kuno et al. 1986). It is therefore possible that semen is not only a vehicle of transport for the virus, but that it increases virus release from infected cells, and increases host susceptibility to infection by decreasing immune cell function.

Local immunosurveillance by NK cells could also be impaired, and an increased susceptibility to neoplastic change result. Prostatic cancer is the second most common neoplasm in men (McGregor and Teper 1978), and cervical cancer is prevalent in women with a high degree of sexual activity and multiple partners (Wynder 1969). HPV infection is strongly associated with the risk of developing cervical cancer (Mitchell et al. 1986) and zur Hausen (1982) has proposed that infected individuals may progress to cervical neoplasia by initiating events such as concomitant HSV infection, or heavy smoking. It is postulated that repeated exposure to seminal plasma and the resulting impaired immunosurveillance by NK cells may also help neoplastic change if repeated or persistant HPV infections are present.

4.8 CONCLUSIONS AND FUTURE STUDIES

Many publications have reported on the immunosuppressive properties of seminal plasma, but the complexity of the material has hampered identification of the factors responsible for this suppression and few characterisation studies have been made. Studies have also been made on cross species systems, but much of the data is difficult to interpret due to the use of bovine serum supplements in culture.

The data presented in this thesis clearly illustrates that seminal plasma components inhibit the function of both NK cells and T lymphocytes, and that the E series prostaglandins are responsible for a major proportion of this suppression. Fractions rich in PGE and 19-OH-PGE suppressed cell function, removal of the prostaglandins removed much of the suppressive activity of the seminal plasma and the immunological properties of purified E-series prostaglandins resembled those of whole seminal plasma.

The findings go someway in explaining the uniquely high concentration of E-series prostaglandins in semen. Concentrations of prostaglandin E are several log times greater than those in an inflammatory situation (Higgs and Salmon 1979), but their presence has not been satisfactorily explained. The interaction of seminal prostaglandins has been frequently examined, but effects on sperm motility are moderate, do not occur in all samples, and have not been demonstrated with mixtures of prostaglandins as they occur in semen (Schlegel *et al.* 1981, Gottlieb *et al.* 1988).

Kelly (1988) suggests the unusually high distribution of prostaglandins in the semen of humans may have evolved to cope with his unique sexual habits. In most species sexual activity is restricted by the receptivity of the female to one part of the ovarian cycle, or additionally, in seasonal breeders, to one part of the year. Man faces no biological restrictions, and the prostaglandins may provide long term protection against sensitisation of the recipient female's immune system to semen. This hypothesis is supported by data from sub-human primates. In the gorilla, the female usually only mates with one male, whereas the chimpanzees and macaque monkeys indulge in multiple matings with the female (Kelly 1988). Although data is limited, these behavioural patterns accord with the considerably lower levels of prostaglandin in the gorilla compared to the chimpanzee and macaque monkey (Kelly et al. 1976).

Continuation of this work will involve the characterisation and identification of the proteins responsible for binding seminal prostaglandins, and the study of the immunological properties of this complex. Further work delineating the differential effects of prostaglandins on lymphocytes and macrophages will also be made. Currently, a study is also underway measuring the PGE and 19-OH-PGE concentration and immunosuppressive activity of semen samples from patients with unexplained infertility, and in normal controls with proven fertility. In a much broader context, it is essential that further work is made on the distribution and function of immune cells in the male and female reproductive tracts.

The results from this study are important and particularly relevant to a number of clinical situations. Further study on the protein-prostaglandin interaction may reveal a novel immunosuppressive agent relevant in the field of transplantation; knowledge of the immunology of the reproductive tract and its secretions are essential if an effective contraceptive vaccine is to be developed; and contribution to our understanding on the transmission of HIV is paramount with the present worldwide mortality from AIDS.

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APPENDIX

ADDRESSES OF SUPPLIERS

1. Amersham International plc
   UK Sales Office
   Lincoln Place
   Green End
   Aylesbury
   Buckinghamshire HP20 2TP

2. Becton Dickinson UK Ltd.
   Between Towns Road
   Cowley
   Oxfordshire OX4 3LY

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   Thornliebank
   Glasgow G46 7TP

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   Lewes
   East Sussex BH7 1LG

5. Corning Medical Ltd.
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   Essex CO9 2DX

6. Cayman Chemicals Ltd.
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   Michigan, USA

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   The Octagon
   High Wycombe
   Buckinghamshire HP11 2HT

8. Flow Laboratories
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   Harefield Road
   Rickmansworth,
   Hertfordshire WD3 1PQ

9. Gelman Sciences Inc.
   600 South Wagner Road
   Ann Arbor
   Michigan 48106 USA

10. Gibco Ltd.
    PO Box 35
    Trident House
    Renfrew Road
    Paisley PA3 4EF

11. LKB
232 Addington Road,
Selsdon, South Croydon
Surrey, CR2 8YD

12. Monojet
Sherwood Medical
St. Louis
MO 63103 USA

13. New England Nuclear Products Ltd.
c/o Dupont UK
Wedgwood Way
Stevenage
Hertfordshire SG1 4QN

14. Pharmacia Ltd.
Pharmacia House
Midsummer Boulevard
Milton Keynes MK9 3HP

15. Scottish Antibody Production Unit
Glasgow and West of Scotland Blood Transfusion Service
Law Hospital
Carluke
Lanarkshire ML8 5ES

16. Sigma Chemical Company
Fancy Road
Poole
Dorset BH17 7NH

17. Skatron AS
Dolasletta 1
Tranby
3401 Lier
Norway

18. Sterilin Ltd.
Lampton House
Lampton Road
Hounslow
Middlesex TW3 4EE

19. Waters (Millipore)
The Boulevard
Ascot Road
CroxleyGreen
Watford
Hertfordshire WD1 8YW

ABBREVIATIONS

AB	AB blood group positive
B cell	Bursa derived lymphocyte
CMV	Cytomegalovirus
CPM	Counts per minute
Con A	Concanavalin A
DEAE	Diethylamino ethyl cellulose
DNA	Deoxyribonucleic acid
DNP-F	2-4-dinitrophenyl-L-lysine-ficoll
EBV	Epstein Barr Virus
EDTA	Ethylenediaminetetra acetic acid
FCS	Foetal calf serum
HB103	HB103 serum free medium
HBSS	Hanks balanced salt solution
HIV	Human immunodeficiency virus
HLA-DR	Human leucocyte antigen class II
HPLC	High pressure liquid chromatography
HPV	Human papilloma virus
HSV	Herpes simplex virus
IL-1	Interleukin 1
IL-2	Interleukin 2
IL-2R	Interleukin 2 receptor
LF	Lyophilised fraction
LPS	Lipopolysaccharide
McAb	Monoclonal antibody
MLR	Mixed lymphocyte reaction
NBCS	New born calf serum
ND	Not done
NK cell	Natural killer cell
NRS	Normal rabbit serum
NS	Not significant
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cells
PHA	Phytohaemaglutinin
PGE	Prostaglandin E
19-OH-PGE	Nineteen hydroxy prostaglandin E
19-OH-PGF	Nineteen hydroxy prostaglandin F
PGF	Prostaglandin F
PMA	Phorbol myristate acetate
PWM	Pokeweed mitogen
R M	Rabbit antimouse
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RPMI	RPMI cell culture medium 1640
SD	Standard deviation
SP	Seminal plasma
TBS	Tris buffered saline
T-cell	Thymus derived lymphocyte
TNP-BA	Trinitrophenyl-*Brucella abortus*
TT	Tetanus toxoid
Zn	Zinc
DAB	Diaminobenzidene

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Human seminal plasma suppresses lymphocyte responses in vitro in serum-free medium

Stanislaw Szymaniec[a], Alison J. Quayle[b], T.B. Hargreave[b] and Keith James[b]

[a]*Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, UL Czerska, 53-114 Wroclaw (Poland) and* [b]*Department of Surgery, University of Edinburgh Medical School, Teviot Place, Edinburgh, EG8 9AG (Scotland)*

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Summary

The in vitro immunosuppressive properties of human seminal plasma have been re-investigated in serum-free medium in view of recent suggestions that the previously observed effects might be dependent on the presence of exogenous serum co-factors present in the culture media. The present studies reveal that low concentrations of seminal plasma can inhibit the ability of peripheral blood leukocytes to lyse K562 target cells in the absence of fetal calf or new-born calf serum. These inhibitory effects could be achieved by pre-incubating the effector cells in seminal plasma at 37°C prior to use in the natural killer cell assay or by incorporating it into the assay system. Additional studies revealed that human seminal plasma could also inhibit the proliferative response of peripheral blood lymphocytes to phytohaemagglutinin in serum-free HB103 medium. These effects were most marked and consistent if the seminal plasma was present throughout the period of culture. Overall, these studies indicate that the previously reported suppressive effects of human seminal plasma in these systems cannot be entirely attributable to cytotoxic factors generated by exogenous serum components.

Key words: *seminal plasma; immunosuppression; in vitro assays; serum-free medium.*

Correspondence to: Dr. K. James.

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Introduction

There have been many reports showing that human seminal plasma can impair the activity of most of the cells of the immune system and also interfere with the generation and activity of a number of the soluble factors involved in the defence against infection (see James and Hargreave, 1984). This has led to the suggestion that while the main purpose of immunosuppressive components in seminal plasma may be to limit autoimmune responses to differentiation antigens on sperm, excessive exposure might also predispose to a number of problems of increasing clinical importance, including prostatitis, epididymitis, malignancies of the urogenital tract and sexually transmitted diseases, including AIDS (e.g. James et al., 1983; James and Hargreave, 1984; McClean et al., 1983; Rees et al., 1986; Tarter et al., 1986).

Recently, however, there have been reports that the ability of human seminal plasma to suppress certain in vitro responses, namely mitogen-induced lymphocyte transformation and natural killer cell activity, is dependent upon the presence in the culture media of bovine serum (Rees and Vallely, 1986). This has led to the suggestion that the immunosuppression observed in these systems is due to factors produced by the interaction of polyamine oxidases in bovine serum with spermine, a major component of seminal plasma. These factors include oxidised spermidine itself (Rees and Vallely, 1986; Allen and Roberts, 1986a,b) and its highly toxic degradation product, acrolein (Allen and Roberts, 1986a,b).

While the ability of the above products to inhibit certain lymphocyte responses in vitro is beyond doubt (e.g. Byrd et al., 1977; Gaugas and Dewey, 1978; Williamson, 1984) the suggestion that certain of the observed immunosuppressive effects of human seminal plasma are due to the action of serum co-factors of bovine origin are of considerable importance as they challenge the possible biological relevance of most of the previous studies in this area. We therefore felt it was important to perform further studies to establish if the in vitro immunosuppressive effects of human seminal plasma were dependent upon the interaction with bovine serum factors. The results of this study clearly indicate that the ability of seminal plasma to inhibit the response of peripheral lymphocytes to phytohaemagglutinin and their ability to lyse K562 target cells is not crucially dependent upon the presence of exogenous serum co-factors.

Materials and methods

The semen samples were obtained from routine laboratory specimens

provided by donors attending the male infertility clinic run by our department. After liquefaction the semen samples were centrifuged at 1000 × *g* for 10 min to remove spermatozoa. Samples were either tested individually (see Table 1) or about 20 samples pooled. The individual samples and pools were sterilised by passage through a Hemming filter and stored at −20 °C until used.

The peripheral blood leukocyte preparations used were derived from fresh whole blood obtained with consent from healthy laboratory staff or routine blood donors. Blood was collected into 10 ml sterile blood collection tubes containing 0.1 ml of a 15% (w/v) solution of EDTA (Monojet, Sherwood Medical, St. Louis, MO, U.S.A.). Leukocyte separations were performed on lymphocyte separation medium (Flow Laboratories, Irvine, U.K.). The cells obtained were washed (×3) with Hank's balanced salt solution and resuspended to the required cell density in RPMI-1640 containing either 10% (v/v) heat-inactivated (56 °C for 30 min) fetal calf serum or new-born calf serum or in HB103 serum-free medium (New England Nuclear, Stevenage, U.K.). The HB103 medium is a modified RPMI-1640 basal medium supplemented with purified human albumin (Cohn Fraction V) and transferrin and also contains selenium and phosphoethanolamine. The total protein content is less than 730 μg/ml. For commercial reasons, precise details of composition are unavailable but it is highly unlikely that this product exhibits polyamine oxidase activity. It should also be noted that when used in the transformation assays the above media were also supplemented with 2 mm L-glutamine, 100 Iu penicillin/ml, 100 μg streptomycin/ml and 5×10^{-5} M mercaptoethanol.

Adherent cell-depleted effector cells for use in the NK cell assay were obtained by further incubation of the above preparations in 75 cm^2 plastic flasks for 20 min at 37 °C.

Full details of the basic NK cell assay used, including the growth and ^{51}Cr-labelling of the K562 target cells, the incubation conditions and the procedure for determining specific chromium release have been described elsewhere (James and Szymaniec, 1985). Further details on pre-incubation conditions, effector target ratios used and the concentration of seminal plasma employed are recorded in the results section.

Mitogen-induced lymphocyte proliferation studies were performed with a wide range of concentrations (0.3—40 μg/ml) of phytohaemagglutinin (Sigma, Poole, U.K.). The cultures which were performed in triplicate on 96-well round-bottomed microtitre plates, contained 1×10^5 cells in 100 μl of serum-free or serum-containing media, 20 μl of mitogen solution and 20 μl of human seminal plasma or media. The final concentration of seminal plasma in culture was 1% (v/v). Following 72 h incubation at 37 °C the cultures were pulsed with 1 μCi/ml tritiated thymidine

(Amersham International, Amersham, U.K.) and harvested 18 h later on a Skatron semi-automatic harvester (Skatron, Lier, Norway). The discs were suspended in scintillant and counted on a β scintillometer. Further details on the pre-incubation of lymphocytes are desribed below.

Cell viability was assessed using the vital dye ethidum bromide acridine orange (Parks et al., 1979).

Results

In our initial studies in serum-free media we examined the inhibitory effects of human seminal plasma from 10 different donors on the lysis of K562 target cells by peripheral blood leukocytes, the seminal plasma being present throughout the 4-h assay period. These studies clearly demonstrated that every sample of seminal plasma examined was capable of inhibiting natural killer cell activity in HB103 serum-free medium (see Table 1).

Additional studies revealed that these inhibitory effects in serum-free media could be achieved with a range of concentrations of seminal plasma, there being some suggestion however that with low doses the effects were more marked in the presence of FCS (Fig. 1).

TABLE 1

The effect of human seminal plasma[a] on NK cell activity in serum-free medium.

Experiment	Donor	% Inhibition	
		HB103 medium	RPMI containing 10% FCS
	1	40.0	64.6
	2	60.0	87.0
A[b]	3	45.5	73.4
	4	67.3	73.2
	5	74.6	71.4
Mean ± S.D.		57.5 ± 13.0[d]	73.9 ± 7.3
	6	79.8	29.6
	7	86.4	81.0
B[c]	8	91.9	99.5
	9	92.2	82.8
	10	85.2	73.7
Mean ± S.D.		87.1 ± 4.6	73.3 ± 23.4

[a]1% v/v.

[b]Effector/target ratio, 12 : 1. Maximum release 39.1%.

[c]Effector/target ratio, 30 : 1. Maximum release 61.1%.

[d]This is just significantly lower ($P < 0.05$) than in serum-containing cultures.

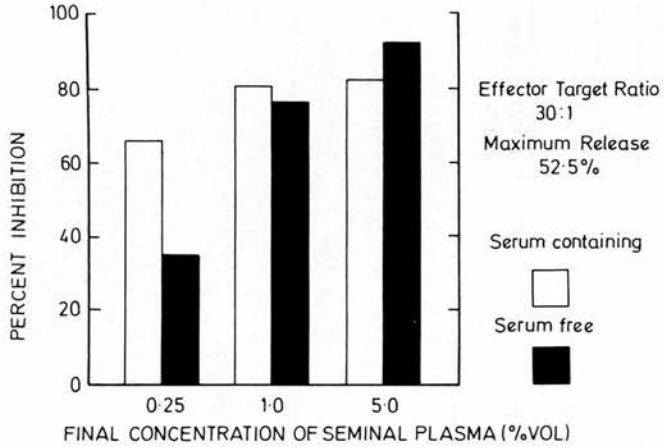

Fig. 1. The effect of different doses of human seminal plasma on natural killer cell activity in serum-free media. Note that effective inhibition is achieved in serum-free media with all doses of seminal plasma. The serum used in these experiments was FCS.

Other experiments were performed to determine the influence of various pre-incubation procedures on the immunosuppressive properties of human seminal plasma in serum-free systems. In these studies the effector leukocytes were pre-incubated at various temperatures and for varying

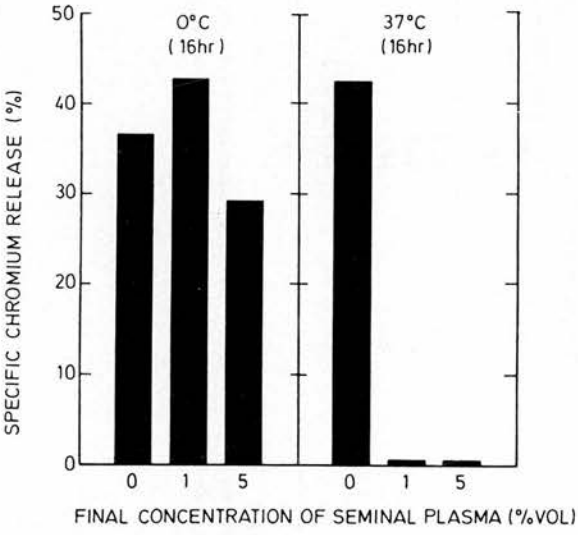

Fig. 2. The influence of pre-incubation temperature on the inhibitory effects of human seminal plasma in serum-free media (HB103). Note that prior treatment of peripheral blood leukocytes in human seminal plasma at 37°C in the absence of bovine serum components drastically impairs natural killer cell activity while treatment at 0°C does not.

TABLE 2

The effect of human seminal plasma[a] on phytohaemagglutinin[b] induced lymphocyte transformation in serum-free medium (HB103). Note that similar inhibitory effects were seen with a range of mitogen doses and peripheral blood lymphocytes from a number of donors.

Experiment[d]	% Inhibition observed[c] following					
	1 h pre-treatment in			Continuous treatment in		
	HB103 medium	RPMI + 10% FCS	RPMI + 10% NBCS	HB103 medium	RPMI + 10% FCS	RPMI + 10% NBCS
1	13.3	12.5	31.9	69.5	93.8	69.5
2	0	69.1	0	93.1	57.0	93.1
3	0	14.4	6.7	88.0	98.0	88.0
Mean S.D.	4.4 ± 6.3	32.0 ± 26.2	12.9 ± 13.7	83.5 ± 10.1	82.9 ± 18.4	75.5 ± 22.4

[a]1% v/v.
[b]5 μg/ml PHA.
[c]Percent inhibition with reference to stimulation observed in PHA stimulated culture without human seminal plasma.
[d]Experiments done on 3 separate occasions with peripheral blood lymphocytes from a single donor.

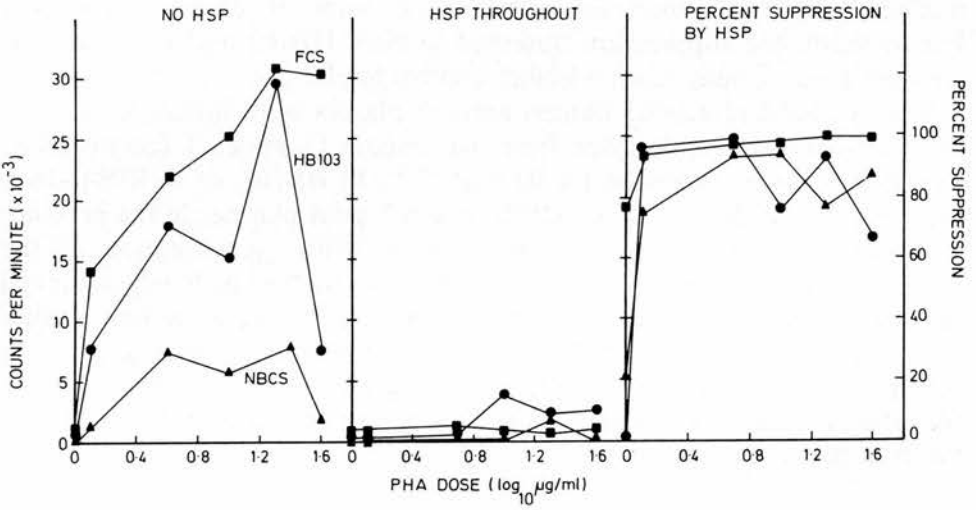

Fig. 3. The effect of human seminal plasma on PHA-induced lymphocyte transformation in serum-free and serum-containing media. Note that human seminal plasma (1% v/v) effectively suppresses the proliferative response to a wide range of doses of phytohaemagglutinin in both serum-free (HB103) and serum-containing media (FCS and NBCS).

periods of time in 1% and 5% (v/v) human seminal plasma in serum-free (HB103) medium. The cells were then extensively washed in serum-free medium prior to testing in the standard K562 lysis assay without the addition of further seminal plasma. It is apparent from Fig. 2 that the ability of human seminal plasma to inhibit natural killer cell activity is drastically influenced by the temperature of pre-incubation. Thus pre-incubation of effector cells at 0°C for 16 h had little effect on their lytic ability when subsequently tested in the standard NK assay. In contrast, pre-incubation at 37°C resulted in complete ablation of their activity. Additional studies revealed that similar inhibitory effects could be achieved in serum-free medium by pre-incubation at 37°C for much shorter periods, namely 1—4 h.

Extensive studies on the effect of human seminal plasma on phytohaemagglutinin-induced lymphocyte proliferation gave results in broad agreement with the above, indicating that marked inhibition could be achieved in the absence of bovine serum components. The results of three separate experiments on lymphocytes from a single donor are summarised in Table 2 while those obtained in one such experiment are illustrated in greater detail in Fig. 3. Throughout these studies it has been apparent that the inhibition achieved in all three media is more dramatic and reproducible when human seminal plasma is present throughout the period of culture. It should also be noted that inhibitory effects in HB103

medium have been observed with a wide range of doses of mitogen. Furthermore, the suppression observed in both HB103 and FCS containing media was usually from a higher control level.

The cytotoxic effects of human seminal plasma were investigated using peripheral blood lymphocytes from six donors (3 male, 3 female). The lymphocytes were incubated for 92 h at 37°C in HB103, or in RPMI-1640 supplemented with 10% FCS, NBCS or autologous plasma, in the presence or absence of 1% human seminal plasma. With the exception of the NBCS-containing cultures, human seminal plasma had little effect on cell survival. In almost all cases survival and viability were similar to that observed in controls with viabilities in most instances exceeding 90%. In contrast, in the presence of NBCS, HSP appeared to be extremely toxic for lymphocytes with few cells surviving culture for 92 h in 4 out of 6 of the donors tested.

Discussion

In the current studies we have demonstrated that human seminal plasma is capable of impairing the activity of cells of the immune system in HB103 medium which contains no bovine serum components. The responses inhibited included NK cell mediated lysis of K562 target cells and mitogen-induced lymphocyte proliferation. These results indicate that the inhibitory effects observed in certain of these in vitro models, by ourselves and others, cannot be attributable solely to the interaction of exogenous bovine serum co-factors with human seminal plasma components such as spermine (Rees and Vallely, 1986; Vallely and Rees, 1986).

At the present time we do not have a satisfactory explanation of the differences between our results in HB103 medium and those previously reported by Vallely and Rees (1986). It is however conceivable that it might be partly due to differences in experimental protocols. In this connection it is interesting to note that Vallely and Rees (1986) incubated their effector cells at 37°C for 18 h prior to use in the NK cell assay. In our hands this leads to enhanced NK cell activity and a markedly reduced susceptibility to inhibition by HSP in the presence of fetal calf serum (Quayle et al., 1987). Further studies in identical experimental models should help resolve this matter.

While our results in HB103 medium are at variance with those previously reported we accept that in certain experimental models the immunosuppressive properties of human seminal plasma may be attributable to the generation of oxidised spermine (Vallely and Rees, 1986; Rees and Vallely, 1986) and its toxic degradation product acrolein (Allen and Roberts, 1986). Indeed, our own cytoxic studies would suggest that culture of

lymphocytes for 48 h or more in HSP in newborn calf serum containing media can result in their death. In contrast, however, little or no cell death was observed in either serum-free or FCS-containing media. It is our contention that a variety of factors and mechanisms are involved and as previously emphasised these may vary from system to system (see James and Hargreave, 1984). Of special interest as far as the NK cell system is concerned are recent reports indicating that the inhibitory effects of human seminal plasma can be directly attributed to 19-hydroxylated E prostaglandins (Tarter et al., 1986). The present studies further support the view that the inhibitory effects of human seminal plasma on human peripheral blood leukocytes cannot be entirely attributable to cytotoxicity. In the first place, prolonged incubation (92 h) of these cells in inhibiting concentrations of HSP in both serum-free and FCS or autologous plasma containing media resulted in little cell death. In view of these results we find it difficult to believe that the inhibitory effects noted in the relatively short term NK cell model can be attributed to the killing of the effector cells, unless they are particularly susceptible. However, in view of our observations that the NK cell activity of peripheral blood leukocytes incubated at 37 °C for 18 h is not readily inhibited by human seminal plasma (Quayle et al., 1987), we feel this is not the case unless this procedure induces phenotypic changes which render the cells refractory. Nevertheless, we would concede that the peripheral blood lymphocytes of certain individuals might be particularly sensitive to seminal plasma, especially if the latter contains antibodies which might cross-react with lymphocytes.

Although differences in immunosuppressive effects of HSP have been noted in serum-free media they do not detract from the possible clinical importance of previous reports in this field but emphasise the need for further studies. The relevance or otherwise of bovine serum co-factors in different experimental systems will only become fully apparent when investigations are performed with purified seminal plasma components and as their mode of action becomes understood. In this connection it is interesting to note that our recent studies indicate that human seminal plasma may inhibit mitogen induced lymphocyte proliferation by impairing the generation of IL-2 receptors (Quayle et al., 1987).

Acknowledgements

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Immunosuppression by seminal prostaglandins

A. J. QUAYLE, R. W. KELLY*, T. B. HARGREAVE & K. JAMES *Department of Surgery, Edinburgh University Medical School and *Medical Research Council, Reproductive Biology Unit, Edinburgh*

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SUMMARY

In this paper we report studies undertaken to determine the contribution of seminal prostaglandins to some of the known immunosuppressive properties of human seminal plasma. Initial studies revealed that fractions of seminal plasma enriched in E series prostaglandins, obtained by reverse phase chromatography, had a pronounced inhibitory effect on the PHA-induced proliferation of peripheral blood lymphocytes and on the NK-cell-mediated lysis of K562 target cells. Additional investigations revealed that similar inhibitory effects could be achieved with purified PGE_2 (10^{-6} to 10^{-9} M) and 19-OH PGE_1 (10^{-6} to 10^{-7} M), both of which are present in uniquely high concentrations in human seminal plasma. In contrast, 19-OH PGF_1 which is found in lower concentrations in semen was slightly stimulatory in proliferative assays and had no effect on NK-cell-mediated cytotoxicity. Removal of the seminal prostaglandins by absorption chromatography resulted in a dramatic decrease in immune suppressive activity. Further studies with fractions obtained by ion-exchange HPLC of desalted seminal plasma indicated that prostaglandins complexed with seminal proteins, and these too were immunosuppressive. The possible relevance of these results to sexually transmitted disease is discussed.

Keywords prostaglandin E seminal plasma AIDS

INTRODUCTION

Seminal-plasma-mediated immunosuppression is well documented and is believed to prevent sensitization of the female to spermatozoa after coitus (Stites & Erickson, 1975; James & Hargreave, 1984; Alexander & Anderson, 1987). The complexity of seminal plasma has hampered the identification of the factor(s) responsible for this suppression and few characterization studies have been reported.

Semen contains four main prostaglandins (PGs) all of which are of the E series; PGE_1, PGE_2, 19-OH PGE_1 and 19-OH PGE_2, with up to 2×10^{-4}M PGE and even higher levels of 19-OH PGE (Templeton, Cooper & Kelly, 1978). The hydroxylated prostaglandins are only found in appreciable concentrations in primate semen (Kelly *et al.*, 1976).

Prostaglandin E is a well known immunoregulatory molecule (Goodwin & Ceuppens, 1983; Wickremasinghe, 1988), but 19-OH PGE has only been reported as an immunosuppressant in a single study (Tarter, Cunningham-Rundles & Koide, 1987). Since the two previous reports implicating prostaglandins as possible immunosuppressive factors in semen have not distinguished between 19-OHPGE and PGE (Tarter *et al.*, 1987; Vallely, Sharrard & Rees, 1988), we have evaluated the contribution of these two types of prostaglandin to mitogen-induced lymphoproliferation and NK-cell-mediated cytotoxicity using reverse-phase fractionated semen and standard prostaglandin preparations. We have also examined the association of prostaglandins in semen with protein components, an observation not previously reported. It is our belief that these findings help explain the known *in vitro* immunosuppressive effects of seminal plasma and, as emphasized previously, may be relevant to sexually transmitted disease (James & Hargreave, 1984).

Correspondence: A. J. Quayle, Department of Surgery, The Medical School, Teviot Place, Edinburgh, EH8 9AG, UK.

MATERIALS AND METHODS

Seminal plasma (HSP)

Semen samples obtained from patients attending the infertility clinic at the Western General Hospital, were centrifuged at 1000 *g* for 10 min to remove spermatozoa and cells, and frozen at −20 °C within 4 h of receipt. Before use samples were thawed and a pool of approximately 30 donors was made. The pool was centrifuged at 80000 *g* for 15 min and the precipitate was discarded.

Reverse phase chromatography

Twenty millilitres of the seminal plasma pool was separated by reverse phase chromatography on a 15 × 300 mm C18 30 μM silica column (Waters, Harrow, UK) with a linear gradient of acetonitrile in water from 0–80%. Sixty 4 ml fractions were collected over a 4 h period. A 0·7 ml aliquot of each fraction was

removed to determine UV absorbance and 0·1 ml was diluted to 1·1 ml with a methyloximating solution and assayed by radioimmunoassay for PGE. The remainder of the fractions were freeze-dried and reconstituted with RPMI-1640 (Gibco, Glasgow, UK). Aliquots were frozen at −20 °C until use.

Anion exchange chromatography

A fraction (2·5ml) of the seminal plasma pool was loaded onto a PD10 Sephadex G25 column (Pharmacia, Uppsala, Sweden) which had previously been washed with 20 ml water. The excluded protein was collected in 3·5 ml water and 2·0 ml of this eluate together with 2·5 μCi of a PGE_2 tracer (Amersham Int., Amersham, UK) was injected into a 7·5 × 75 mm DEAE 5PW HPLC column (Waters). The column was equilibrated with 20 mM ammonium formate (pH 7·6) (buffer A) and eluted at 3 ml/min with a gradient to 100% of buffer B (1·0 M ammonium formate). Protein distribution was determined by UV absorption at 280 nM and 2-min fractions were collected. Distribution of labelled PGE was assessed by scintillation counting. The PGE content of each fraction was determined by mixing 200 μl aliquots of each fraction with methyloximating solution and measuring after suitable dilution in the PGE_2 radioimmunoassay. The remainder of each fraction was freeze-dried and reconstituted with RPMI-1640 (Gibco). Aliquots were frozen at −20°C until use.

Delipidation of semen by absorption chromatography

Two millilitres of seminal plasma was delipidated by passage through a C18 reverse-phase cartridge (Seppak, Waters) which had been primed with methanol. The eluate was found to have negligible PGE and 19-OH PGE levels and was stored at −20 °C until use.

Radioimmunoassay (RIA)

Radioimmunoassays were performed on PGs protected as the methyloximes, using an approach described previously where the antisera were raised against the methyloximated PGE (both E and 19-OHE) and methyloximated standards were used to create the standard curves (Kelly *et al.*, 1984). For the 19-OH PGE assay the PG used to raise the antiserum was extracted from human semen and the antiserum cross-reacted equally with 19-OH PGE_1 and 19-OH PGE_2. The cross-reactivity against PGE_2 and PGE_1 was less than 1% although there was appreciable (5%) cross-reactivity with 19-OH PGF. This latter was not a problem given the far lower amounts of 19-OH PGF found in semen (Templeton *et al.*, 1978). In the PGE_2 assay the cross-reactivity against PGE_1 was 26% and against 19-OH PGE_1 it was 1%. The tracers used were a ^{125}I-labelled peptide coupled through proline to PGE_2 methyloxime for the assay, and a similar conjugate with tyrosine methyl ester for the 19-OH PGE methyl oxime assay. Separation of bound and free tracer was achieved by magnetic separation of second antibody coupled to magnetic latex particles (Amerlex, Amersham International).

Peripheral blood mononuclear cells (PBMC)

Blood samples were obtained with consent from laboratory staff. Blood was collected into EDTA, and PBMC isolated by gradient centrifugation on lymphocyte separation medium (Flow Labs, Irvine, UK). The cells obtained were washed three times with HBSS (Gibco) and resuspended in RPMI-1640 supplemented with 10% human AB serum (Sigma, Poole), 2 mM L-glutamine, 100 iu/ml penicillin, 100 μg/ml streptomycin and 5×10^{-5}M mercaptoethanol.

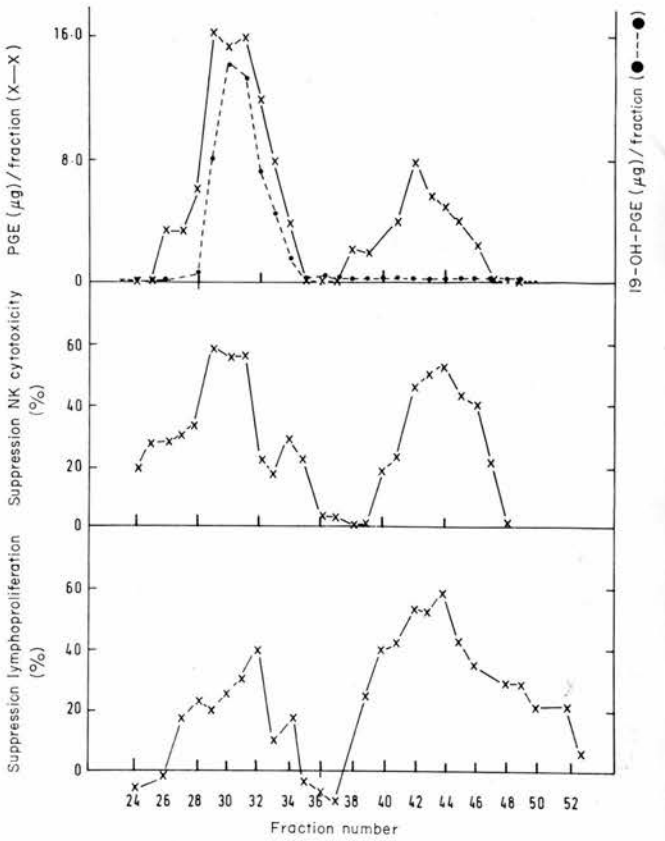

Fig. 1. Distribution of PGE, 19-OH PGE and inhibitory activity to NK cell cytotoxicity and lymphoproliferation in fractions from reverse-phase-separated HSP.

Prostaglandin standards

PGE_2 was obtained from Sigma and 19-OH PGE_1 and 19-OH PGF_1 from Cayman Chemicals (Ann Arbor, Michigan, USA).

Lymphoproliferations

Cells were adjusted to 1×10^6/ml and 0·1 ml aliquots dispensed in triplicate in 96-well round-bottomed plates. PHA (Sigma) (0·125 to 20 μg/ml), and medium alone, RPMI diluted HSP, fractions or PG standards were added in 20 μl. The cultures were pulsed after 72 h with 1 μCi/well of ^{3}H-thymidine (Amersham) and harvested 18 h later on a Skatron cell harvester. Incorporated thymidine was measured in a scintillation counter.

NK cell assay

NK cell activity was assessed by measuring the lysis of ^{51}Cr-labelled K562 target cells by adherent-cell-depleted PBMC. The method has been described elsewhere (James & Szymaniec, 1985). An effector target cell ratio of 25:1 was used.

RESULTS

Reverse phase chromatography

Radioimmunoassay of the fractions obtained from the C18 reverse phase HPLC revealed two peaks of prostaglandin E

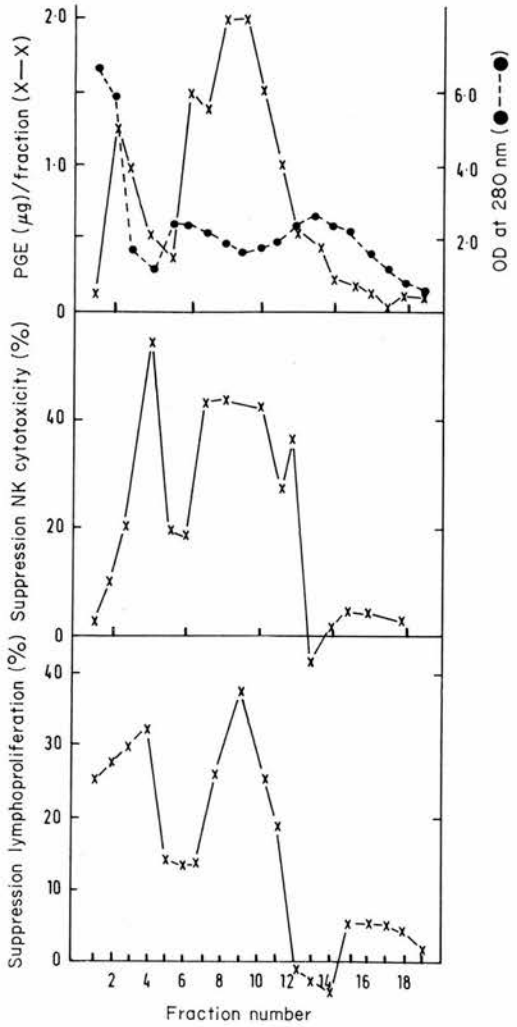

Fig. 2. Distribution of PGE-like material, protein and inhibitory activity to NK cell cytotoxicity and lymphoproliferation in fractions from a desalted HSP pool run on a DEAE HPLC column.

activity between fractions 26–34 and fractions 38–46 (Fig. 1a). Radioimmunoassay demonstrated that 19-OH PGE eluted almost totally in the first peak (Fig. 1a). However, the composition of the first peak of E-like material is almost certainly heterogeneous and possible contributing components are discussed below. The broad peak shape due to large particle size and a wide column was an unavoidable aspect of the chromatography of whole seminal plasma. In both the NK cell (Fig. 1b) and lymphocyte transformation (Fig. 1c) assays, suppression of cell function correlated with the two peaks of prostaglandin activity. The interim fractions (35–37) had no effect in the NK cell assay and were slightly stimulatory in the proliferative assay. An initial assay (data not shown) has revealed 19-OH PGF activity in these fractions but this extends also to the higher fractions. All anti-proliferative effects of these reverse phase fractions, ion-exchange fractions and pure preparations were most effective at sub-optimal PHA doses, a phenomenon noted previously by other workers (Goodwin & Ceuppens, 1974). The results presented are those obtained with a suboptimal PHA concentration for the particular donor.

Table 1. The effect of seminal plasma and standard PG preparations on lymphoproliferation and NK cell activity

Prostaglandin concentration	PBMC response to 2·5 μg/ml PHA ([^{3}H]-TdR uptake, ct/min)*	NK-cell-mediated cytotoxicity (% ^{51}Cr release)*
Control	23311 (100·0)	43·7 (100·0)
HSP 2% (V/V)	3260 (14·0)	7·9 (18·1)
10^{-6}M PGE_2	7144 (30·6)	17·9 (41·0)
10^{-7}M PGE_2	8248 (35·4)	22·2 (50·8)
10^{-8}M PGE_2	12158 (52·2)	29·8 (68·2)
10^{-9}M PGE_2	16138 (69·3)	38·1 (87·2)
10^{-6}M 19-OH PGE_1	9341 (40·1)	18·9 (43·2)
10^{-7}M 19-OH PGE_1	17771 (76·2)	28·7 (65·7)
10^{-8}M 19-OH PGE_1	27559 (118·2)	27·8 (63·6)
10^{-9}M 19-OH PGE_1	27541 (118·2)	34·4 (78·7)
10^{-6}M 19-OH PGF_1	25303 (108·5)	39·3 (89·9)
10^{-7}M 19-OH PGF_1	24463 (104·9)	39·7 (90·8)
10^{-8}M 19-OH PGF_1	24454 (104·9)	42·4 (97·0)
10^{-9}M 19-OH PGF_1	23827 (102·2)	42·6 (97·5)

* Standard deviations were below 15% of the mean value, and are not given.

Numbers in parentheses are percentage of response observed compared to the control culture (100%) set up in the absence of PG or HSP.

Table 2. A comparison of immunosuppression by seminal plasma and delipidated (DL) seminal plasma

Seminal plasma	PBMC response to 1·25 μg/ml PHA (^{3}H-TdR uptake, ct/min)*	NK-cell-mediated cytotoxicity (% ^{51}Cr release)*
Control	22596 (100·0)	24·8 (100·0)
HSP 0·5% (V/V)	ND	18·9 (76·2)
HSP 2% (V/V)	4871 (21·6)	9·4 (37·9)
HSP (DL) 0·5% (V/V)	ND	24·3 (98·0)
HSP (DL) 2% (V/V)	16828 (74·5)	22·7 (91·5)

* Standard deviations were below 15% of the mean value, and are not given.

Numbers in parentheses are percentage of response observed compared to the control culture (100%) set up in the absence of HSP.

ND not done.

Ion exchange chromatography

The low molecular weight of prostaglandins (approximately 350 Daltons) would suggest that all such components would be lost in the desalting step. However, separation of the excluded proteins from a Sephadex G25 column on a DEAE HPLC column, and subsequent RIA of the fractions demonstrated two peaks of PGE-like activity between fractions 2–4 and 6–12 (Fig. 2a). The PGE_2 tracer labelled with tritium was detected between fractions 6–11 with a peak at fraction 7, indicating that radiolabelled PG did not necessarily co-elute with the radioimmunoassayable PG.

Suppression of both NK cell activity and lymphocyte proliferation correlated with PGE like activity (Fig. 2b & 2c). The small peak of suppressive activity (< 10%) in fractions 15–

17 has not so far been explained. The results from this procedure clearly demonstrate that prostaglandins of the E series bind to seminal proteins, and that such complexes are actively suppressive.

Standard preparations of prostaglandins

In an attempt to establish further the contributions of the prostaglandins to the immunosuppressive properties of human seminal plasma, studies were performed with purified preparations. Three standard preparations of prostaglandins were used in the two assays; PGE_2, 19-OH PGE_1 and 19-OH PHF_1, which is found in lower concentrations in human semen.

The proliferative response of PBMC to a suboptimal concentration of mitogen (Table 1) was suppressed by all concentrations of PGE_2 (10^{-6} to 10^{-9}M) and the two higher concentrations (10^{-6} and 10^{-7}M) of 19-OH PGE_1; 19-OHF_1 was slightly stimulatory. These effects were reduced at optimal PHA concentrations (results not given). PGE_2 and 19-OH PGE_1 also suppressed NK-cell-mediated lysis of K562 target cells at all concentrations used (10^{-6} to 10^{-9}M), while 19-OH PGF_1 had a small (< 10%) inhibitory effect at 10^{-6} and 10^{-7} M (Table 1). Whole seminal plasma (PGE level 4·34 μg/ml), when used in culture at 2% (V/V), suppressed NK cell and lymphocyte function by approximately 80%. Further support for the immunosuppressive role of seminal prostaglandins was obtained using prostaglandin-depleted seminal plasma (PGE level 12·4 ng/ml). Seminal plasma delipidated by passage through a lipid extraction column retained a proportion of suppressive activity against lymphoproliferation (approximately 25%), but was ineffective against NK-cell-mediated-cytotoxicity (Table 2).

DISCUSSION

Seminal plasma is reported to suppress the in-vitro responses of T and B lymphocytes, NK cells, neutrophils, and cells of the monocyte-macrophage lineage, and to interfere with complement activation (James & Hargreave, 1984; Alexander & Anderson, 1987). Although there is a paucity of studies isolating and examining the character of the active substances, suggested mediators of this suppression include zinc and zinc/peptide complexes (Chvapil *et al.*, 1977; Stankova *et al.*, 1976), transglutaminase (Mukherjee *et al.*, 1983), Fc binding protein (Witkin *et al.*, 1983), pregnancy associated protein A (Bischof *et al.*, 1982) and other unspecified high molecular weight proteins (Prakash, Coutinho & Moller, 1976; Lord, Sensabaugh & Stites, *et al.*, 1977) as well as prostaglandins (Tarter *et al.*, 1986; Vallely *et al.*, 1988).

The studies presented here clearly implicate prostaglandins E and 19-OHE as major contributors to seminal-plasma-induced suppression of T cell responses to mitogen and NK-cell-mediated cytotoxicity. This confirms recent reports that seminal prostaglandins may inhibit NK-cell-mediated cytotoxicity (Tarter *et al.*, 1986; Vallely *et al.*, 1988) and has extended their initial observations in a number of ways by clearly demonstrating that both PGE and 19-OH PGE individually can suppress NK-cell-mediated cytotoxicity, that they also inhibit lymphoproliferation and that a substantial amount of the inhibitory effect of human seminal plasma may be due to protein bound prostaglandins. In this study the protein-prostaglandin complex may contribute to the composition of the first peak of the reverse-phase chromatography (Fig. 1), as both immunosuppressive activity and PGE-like material eluted ahead of the 19-OH PGE peak and this area of the chromatogram corresponded with the tail of the protein elution (data not shown).

The degree to which PGs account for all the immunosuppressive activity in semen is the subject of further research, but delipidated seminal plasma samples failed to inhibit the NK cell activity and only exerted a small effect on lymphocyte proliferation. These results suggest that prostaglandins might be entirely responsible for the inhibitory effects observed in the NK cell assay but that additional factors could contribute to the inhibition of lymphocyte proliferation. Furthermore our studies have suggested that prostaglandins bind to seminal protein(s), a phenomenon not previously reported. Preparations of these bound prostaglandins also show immunological activity; this may be due to the release of the prostaglandin or may reflect activity of the complex itself. The presence of the prostaglandin-protein complexes leads us to speculate that the suppression attributed in earlier studies to proteins was actually due to prostaglandin-protein complexes.

These observed immunosuppressive effects appear to be specific to the E series since we show 19-OH PGF to be inactive (Table 1). Studies of synthetic prostaglandins show that PGE and 19-OH PGE have comparable activity in the NK cell assays but PGE is more effective at suppressing the PBMC proliferative response to mitogen.

PGE is produced by many cells, including macrophages, but not lymphocytes. The action of PGE in controlling cell function is by a paracrine mechanism raising intracellular cAMP concentrations through receptors coupled to adenyl cyclase via G proteins (Smith *et al.* 1987). Earlier studies demonstrated suppression of NK activity and of T cell proliferation, lymphokine production and cytotoxicity (Goodwin & Ceuppens, 1974). More recently PGE has been shown primarily to exert its antiproliferative effects through inhibition of IL-2 production (Walker *et al.*, 1983, Mary *et al.*, 1987; Vercammen & Ceuppens, 1987). The expression of receptors for IL-2 (Vercammen & Ceuppens, 1987) and transferrin (Chouaib *et al.*, 1987) may also be inhibited. In our laboratory whole seminal plasma and standard PGE preparations have been found to suppress IL-2 R expression (Quayle *et al.*, 1987) and IL-2 production (unpublished data).

The presence of prostaglandins in semen, many log concentrations above those found in peripheral blood or in an inflammatory situation (Higgs & Salmon, 1979), has attracted interest and speculation, but their presence has not been satisfactorily explained. The interaction of the seminal prostaglandins with spermatozoa has been examined frequently (Schlegel *et al.*, 1981; Gottlieb *et al.*, 1988), but effects on sperm motility are moderate, do not occur in all samples, and have not been demonstrated with mixtures of prostaglandins as they occur in semen. In addition an action on sperm is not obviously compatible with the species distribution of the seminal PGs. The physiological relevance of prostaglandin-mediated suppression of immune responses in the female is attractive as high prostaglandin concentrations would be necessary to ensure total paralysis of sensitization. There is also evidence to suggest that some men with unexplained infertility have low seminal prostaglandin levels (Bygdeman *et al.*, 1970).

We believe that seminal plasma immunosuppression may also be a co-factor in the etiology or pathogenesis of a number of

genito-urinary tract diseases. Sexually transmitted bacteria such as *N. gonorrhoea* and viruses such as herpes, papillomavirus and HIV are causing increasing morbidity and mortality and it is imperative that more is understood about factors involved in the transmission and establishment of these infections. HIV for example has been a high profile disease in homosexual men and epidemiological evidence has shown that passive anal intercourse is the lifestyle factor with the greatest risk of infection (Marmor, 1985). Animal studies have shown that PGE_2 metabolites peak in blood after rectal insemination of semen (Alexander *et al.*, 1986) and the same procedure suppresses systemic immune responses (Richards, Bedford & Witkins, 1984). Furthermore, studies *in vitro* have demonstrated that the MT4 T cell line infected with HIV produces 2·5 times more virus than control cells when exposed to 1·7 μg/ml PGE_2, which is a concentration far lower than that in semen (Kuno *et al.*, 1986). We anticipate that further studies will help elucidate the role of seminal prostaglandins in disease and also improve our understanding of reproductive processes.

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